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ACADEMIC DISSERTATION
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PunaMusta Oy – Yliopistopaino
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To my family

ABSTRACT

Rotavirus (RV) is one of the major causes of acute viral gastroenteritis in children. Symptoms include fever, vomiting and watery diarrhea which may lead to fatal dehydration. Before the development of RV vaccines and their introduction into National Immunization Programs, RV gastroenteritis caused more than 400,000 deaths each year globally. RV vaccines are currently used in more than 90 countries, and the number of deaths has decreased to a third, but RV still remains as one of the main causes of mortality in children under five years of age. The majority of the fatalities occur in low-to-middle income countries, whereas in developed countries, RV is the cause of a substantial medical and financial burden. After the introduction of vaccines, an increase of diversity and changes in circulating RV strains have been reported globally.

RotaTeq consists of five human-bovine reassortant RVs (G1P[5], G2P[5], G3P[5], G4P[5] and G6P[8]) on bovine G6P[5] backbone. The shedding of RotaTeq vaccine viruses was originally reported to occur at a low level but more recent studies have shown higher rates of shedding. In early studies, the duration of shedding ranged from a week, even up to hundreds of days in immunocompromised children. Nevertheless, the characteristics of RotaTeq vaccine strain shedding have not been studied thoroughly. Similarly, the formation and potential higher virulence of vaccine-derived double-reassortant G1P[8] (vdG1P[8]) is mainly unknown.

This thesis focused on studying the characteristics of RotaTeq vaccine virus shedding in children without symptoms of acute gastroenteritis. First, stool samples collected from children hospitalized due to a respiratory tract infection between 2009 and 2011 were studied. The study showed that prolonged asymptomatic shedding was more common than previously expected. The occurrence of shedding was highly associated with vaccine genotype G1 as it was detected in 93 % of the cases. Half of the children shed for more than 14 days, and the longest duration of shedding was 84 days after the third dose of the RotaTeq vaccine.

Secondly, in a prospective study, 301 children received the RotaTeq vaccine at their respective child welfare clinic according to the Finnish schedule at the ages of two, three and five months. RotaTeq vaccine strains were detected in the stools of 93 % of the children 5-10 days after the first dose of the vaccine. Of those children,

20 % continued to shed until the third dose of the vaccine, whereas only two children were detected with the vaccine strain in stools three months after the third dose. The study confirmed G1 to be highly prevalent in shedding: it was found in 82 % of the samples taken after the first dose, it was the only genotype found in long-term shedding continuing up to eight months of age. Children who became long-term shedders were found with more severe symptoms after the first dose of the vaccine, whereas similar association was not found when comparing the possible vdG1P[8] combination to other genotypes. Altogether these results suggest that the five-strain-containing RotaTeq vaccine functions in a similar manner as the single human G1P[8] strain vaccine, Rotarix.

RV was added as part of the microbe strain collection in Finland in 2013, and all laboratory confirmed RV cases from the entire country covering all age groups were collected for genotyping. This material was used to study the long-term effects of the RotaTeq vaccine on circulating RV genotypes in a high vaccine coverage setting for five consecutive RV seasons from September 2013 to August 2018. The total number of RV cases remained stable and at a low level throughout the follow-up, indicating that the RotaTeq vaccine has remained highly efficacious. In children, diversification and redistribution of circulating genotypes, similar to other RotaTeq-using countries, was detected, as G1P[8], G2P[4] and G4P[8] were replaced by G12P[8], G9P[4] and G9P[8]. Two distinct age clusters were also identified, as in addition to children, RV was detected in the elderly more frequently than expected. The genotypes of this age group differed from those of the children, as G2P[4] was predominant during most of the follow-up period. The changes in the circulating genotypes were detected in the elderly a season later, suggesting transmission of the disease from children to older age groups. Overall, this period is long enough to suggest that wild-type RVs cannot be eliminated from circulation even with a high-coverage vaccination using a highly efficacious live oral RV vaccine.

TIIVISTELMÄ

Rotavirukset ovat merkittävimpiä ripulitaudin (gastroenteriitin) aiheuttajia lapsilla. Pahimmillaan tauti voi johtaa vakavaan kuivumaan ja kuolemaan, ja ennen nykyisten rokotteiden käyttöönottoa rotavirusten aiheuttama gastroenteriitti johti vuosittain yli 400 000 lapsen kuoleman maailmanlaajuisesti. Merkittävin osa kuolemaan johtavista tautitapauksista esiintyy kehitysmaissa. Vaikka rotavirusrokotteita on otettu käyttöön yli 90 maassa ja rotavirukseen liittyvien kuolemien määrä on laskenut kolmannekseen, rotavirukset ovat silti edelleen yleisin kuolinsyy alle 5-vuotiailla lapsilla. Rotavirusrokotteiden käyttöönoton jälkeen on havaittu, että kiertävät rotaviruskannat ovat muuntuneet ja aiemmin harvinaiset rotaviruskannat ovat yleistyneet.

Suun kautta otettava rotavirusrokote RotaTeq, koostuu viidestä ihmisen ja vasikan rotaviruksista muodostetuista yhdistelmistä (G1P[5], G2P[5], G3P[5], G4P[5] ja G6P[8]). Ennen rokotteiden käyttöönottoa tehdyissä tutkimuksissa kyseisten rokotevirusten erityksen ulosteisiin havaittiin olevan vähäistä, mutta myöhemmissä tutkimuksissa erityksen on huomattu olevan aiemmin luultua yleisempää. Rokotusta seuraavan rokotevirusten erityksen kesto on tutkimuksesta riippuen vaihdellut viikosta kuukauteen, mutta pitkittynyttä, jopa useiden satojen päivien kestoista eritystä on raportoitu immuunipuutteisilla lapsilla. Rokotevirusten erittymisen kliininen merkitys on kuitenkin jäänyt epäselväksi. Erityksen on yhdistetty niin kutsuttu rokoteperäinen kaksoisyhdistelmä G1P[8] virus, jossa rokotteen kaksi kantaa G1P[5] sekä G6P[8] ovat risteytyneet keskenään. Kyseisen viruksen on esitetty omaavan muita kantoja suuremman taudinaiheuttamiskyvyn.

Väitöskirjan tavoitteena oli tutkia RotaTeq-rokotteen sisältämien viruskantojen eritystä lapsilla. Ensimmäisessä tutkimuksessa rokotevirusten pitkäkestoisen erityksen havaittiin olevan aiemmin esitettyä yleisempää ja esiintyvän jopa lapsilla, jotka olivat joutuneet sairaalahoitoon hengitystieinfektion vuoksi ilman maha-suolikanavan oireita. Aineistossa rokotevirusten eritys yhdistyi selkeästi yhteen rokoteviruksista, sillä genotyyppi G1 oli löydöksenä 93 % tapauksista (N=30). Puolet lapsista eritti rokotevirusta ulosteisiin yli 14 päivää edellisestä rokotuksesta, pisimmillään eritys jatkui jopa 84 päivää rokotuksesta.

Rokotevirusten erityksen tarkempaa tutkimista varten toteutettiin erillinen tutkimus, johon osallistui yhteensä 301 lasta, jotka saivat RotaTeq-rokotteen kansallisen rokotusohjelman mukaisesti (2, 3 ja 5kk iässä). Rokotevirusten eritystä ulosteisiin havaittiin jopa 93%:lla lapsista 5-10 päivää ensimmäisen rokoteannoksen jälkeen. Erityksen yleisyys väheni ajan kuluessa ja enää noin viidennes lapsista jatkoi eritystä juuri ennen kolmatta rokoteannosta otetuissa näytteissä. Pitkittynyttä rokoteviruksen eritystä kolmen kuukauden kuluttua viimeisestä rokoteannoksesta eli noin kahdeksan kuukauden ikään saakka, havaittiin kahdella lapsella. Tutkimus varmisti aiemman tutkimuksen löydöksen G1 genotyypin merkittävyydestä rokotevirusten erityksessä, sillä kyseinen genotyyppi havaittiin 82 %:ssa ensimmäisen rokoteannoksen jälkeen kerätyistä näytteistä, ja ainoana genotyyppinä pitkittyneessä rokoteviruksen erityksessä. Rokoteviruksen eritystä käsittelevät tutkimukset viittaavat siihen, että käytännössä viittä yhdistelmärotavirusta sisältävä RotaTeq-rokote käyttäytyy elimistössä yhden rotaviruskannan G1P[8] sisältävän rokotteen (Rotarix) tavoin.

Vuonna 2013 rotavirus lisättiin osaksi tartuntatautiasetuksessa ilmoitettavia mikrobilöydöksiä. Tähän liittyen kaikki maan kliiniset laboratoriot ovat olleet velvoitettuja toimittamaan positiiviseksi toteamansa rotavirusnäytteet jatkotutkimuksiin genotyypitystä varten. Kyseinen materiaali mahdollisti tutkimuksen RotaTeq rokotteen pitkäaikaisvaikutuksista Suomessa kiertäviin rotavirus genotyyppeihin sekä rotavirustautitaakkaan. Tutkimusjakso alkoi syyskuussa 2013 ja jatkui elokuun 2018 loppuun. Rotaviruksen aiheuttaman tautitaakan havaittiin pysyneen pienenä, merkkinä rokotteen tehon säilymisestä vielä lähes kymmenen vuotta käyttöönoton jälkeen. Kiertävissä rotaviruskannoissa havaittiin selkeää monipuolistumista sekä aiemmin yleisten rotaviruskantojen korvautumisen uusilla Suomessa aiemmin harvinaisilla rotaviruskannoilla, erityisesti lasten tautitapauksissa. G1P[8], G2P[4] sekä G4P[8] korvaantuivat G12P[8], G9P[4] ja G9P[8] kannoilla. Vastaava ilmiö on havaittu myös muissa RotaTeq-rokotetta käyttävissä maissa. Tämän lisäksi, tautitapaukset osoittautuivat keskittyvän selkeästi kahteen ikäryhmään ja tautitapauksia havaittiinkin odotettua enemmän lasten lisäksi yli 70-vuotiailla ikäihmisillä. Toisinkuin lapsilla, kyseisessä ikäryhmässä tautitapaukset olivat useasti G2P[4] rotaviruskannan aiheuttamia. Ikäihmisten rotaviruskannat muuttuivat yhden kauden jäljessä lapsiin nähden, joten tämän perusteella voidaankin ajatella lasten toimivan lähteenä ikäihmisten rotavirustautitapauksille. Tutkimuksen yhteenvetona voidaan todeta, että korkeasta rokotekattavuudesta huolimatta, nykyisillä eläviä viruksia sisältävillä rotavirusrokotteilla rotavirustaudin hävittäminen ei ole mahdollista.

CONTENTS

ABBREVIATIONS

AGE	Acute gastroenteritis
ASC	Antibody secreting cell
DLP	Double-layered particle
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
Ig	Immunoglobulin
INF	Interferon
IS	Intussusception
mAb	Monoclonal antibody
NIP	National Immunization Program
NSP	Non-structural protein
REST	Rotavirus Efficacy and Safety Trial
RT-PCR	Reverse transcription-polymerase chain reaction
RTI	Respiratory tract infection
RV	Rotavirus
RVGE	Rotavirus gastroenteritis
THL	National Institute for Health and Welfare
VE	Vaccine efficacy
VP	Viral protein
vdG1P[8]	Vaccine-derived double-reassortant

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I Markkula J, Hemming M, Vesikari T. Detection of vaccine-derived rotavirus strains in nonimmunocompromised children up to 3-6 months after RotaTeq vaccination. Pediatric Infectious Diseases Journal. 2015 Mar; 34(3):296-8.

II Markkula J, Hemming-Harlo M, Vesikari T. Shedding of oral pentavalent bovine-human reassortant rotavirus vaccine indicates high uptake rate of vaccine and prominence of G-type G1. Vaccine. Accepted 4 Dec 2019.

III Markkula J, Hemming-Harlo M, Salminen M, Savolainen-Kopra C, Pirhonen J, al-Hello H, Vesikari T. Rotavirus epidemiology 5-6 years after universal rotavirus vaccination: persistent rotavirus activity in older children and elderly. Infectious Diseases (Lond). 2017 May;49(5):388-395.

IV Markkula J, Hemming-Harlo M, Savolainen-Kopra C, al-Hello H, Vesikari T. Continuing rotavirus circulation in children and adults despite high coverage rotavirus vaccination in Finland. Journal of Infection. Article In Press. Accepted 26 Sep 2019.

1 INTRODUCTION

Rotavirus (RV), a viral particle with the appearance of a wheel (Latin: *rota* = wheel), was detected for the first time in 1973 in the electron microscopy of duodenal biopsies and stool samples obtained from small children with acute gastroenteritis (AGE) symptoms. The novel virus was found and reported almost concomitantly by Bishop et al.(1) in Australia and by Flewett et al.(2) in the UK. However, the virus was discovered already ten years earlier in animals suffering from diarrhea(3,4).

After its discovery in humans, the significance of the virus was understood as it showed to be the major causative agent of seasonal epidemics of diarrhea in small children, causing a high number of hospitalizations and deaths annually. It was estimated that on the verge of the implementation of the current RV vaccines at the beginning of 2000, RV gastroenteritis (RVGE) caused 450,000 deaths in children under five years of age globally, with the death toll being greater in developing countries(5). However, mortality was even greater prior to the development of oral rehydration therapy and its spread to developing countries. The significant burden of the disease inspired studies on the pathogen, and the development of RV vaccines began already in the 1980s with a Jennerian approach using animal and attenuated human RV strains as the first vaccine templates. The development took nearly 20 years until Rotashield, the first RV vaccine, was licensed and taken into use in the US in 1998. Unfortunately, the vaccine was soon suspended and eventually withdrawn due to increased risk for intussusception (IS)(6). Half a decade later, after extensive safety and efficacy trials, the current RV vaccines, RotaTeq and Rotarix, were licensed. These vaccines are presently used globally, and the number of deaths due to RV has decreased drastically(7). Finland adopted the RotaTeq vaccine in September 2009, and since then, outpatient visits and hospitalizations due to RV have decreased by 90 %(8,9).

2 REVIEW OF LITERATURE

2.1 Biology of rotavirus

2.1.1 Structure of the virion and rotavirus genomics

RVs are non-enveloped double-stranded ribonuclease acid (dsRNA) viruses with an icosahedral protein capsid. The capsid consists of an outer, intermediate and inner layer (Fig. 1). The size of an infectious triple-layered particle is around 100 nm. The RV genome consists of 11 segments of dsRNA contained within the inner core layer. Six of the genome segments code for six structural viral proteins (VP1-4, VP6-7) and the remaining five segments for six non-structural proteins (NSP1-6). Gene segment 11 encodes for both NSP5 and NSP6.

The structure of RV has been studied using cryo-electron microscopy and x-ray crystallography. The inner core layer consists of 120 molecules of the scaffolding protein, VP2 (2690 bp, 102 kDa), into which 12 replication enzyme complexes are attached(10). The complexes are formed of a viral RNA-dependent RNA polymerase, VP1 (3302 bp, 125 kDa), and a capping enzyme, VP3 (2591 bp, 88 kDa)(11,12). On top of the VP2 core is the intermediate layer formed by 780 VP6 (1356 bp, 45 kDa) protein units in the form of trimers that interact with both the inner and outer layer(13). Highly conserved VP6 is the most abundant protein in the RV virion, and it stabilizes the triple-layered particle structure by binding the VP2 and VP7 layers together(10,14). The protein also has antigenic and immunogenic properties(15–17). The calcium-binding glycoprotein VP7 (1062 bp, 37 kDa) particles are arranged into 260 trimers on top of the intermediate VP6 layer(18). Protease-sensitive attachment protein VP4 (2362 bp, 87 kDa) trimers form 60 spike structures that are attached to the VP6 layer at their base and held in place by the surrounding VP7 trimers(19,20). VP7 and VP4 form the outer layer of the virion and both also induce neutralizing antibodies(18,19). During cell entry, intestinal trypsin-like proteases cleave the VP4 protein by proteolysis into two polypeptides, VP5* (stalk and basis) and VP8* (head of the spike) which remain associated in the virion(20).

The RV protein capsid has three different types of channel, of which type I channels are formed by VP6 and VP2 units; they function as exit routes from the virion for the transcribed messenger RNA (mRNA)(21). Type II channels are formed of VP6, and they are located around the type 1 channels. The 60 VP4 spike proteins are on the edge of these channels(22–24). Type III channels are also located in the intermediate VP6 layer in the form of hexamers. All three channels function as binding sites for human VP6 antibodies(24).

Non-structural proteins are located inside the virion, and they each have a specific role in RV replication and pathogenesis. NSP1 (1581 bp, 58 kDa) affects the innate immune system by down-regulating the interferon (INF) system, and it delays cell death by degrading pro-apoptotic proteins at the beginning of RV replication(25,26). NSP2 (1059 bp, 35 kDa) has the ability to bind to single-stranded RNA non-specifically(27), to destabilize nucleic acid helix structure(28), and has also several enzymatic activities(29,30). The NSP2 and NSP5 complex forms the viroplasm in the infected cells in which RV replication occurs(31). NSP5 (667 bp, 21kDa) is a serine- and threonine-rich dimer-forming protein that binds to both single- and double-stranded RNA, but possible other specific functions of NSP5 remain unclear(32,33). NSP6 (278 bp, 12 kDa) is encoded by the same segment 11 as NSP5, and it is known to interact with NSP5; like NSP5, the precise role of NSP6 is still unknown(34). NSP3 (1074 bp, 36 kDa) facilitates mRNA translation and suppresses host protein synthesis(35,36). NSP4 (751 bp, 20 kDa) is the only non-structural protein without an RNA-binding function. The protein is multi-functional as it modulates the intracellular accumulation and cellular distribution of viral proteins in the viroplasm, and therefore it is essential in controlling RV transcription and replication(37,38). NSP4 has also been shown to act as a viral enterotoxin(39).

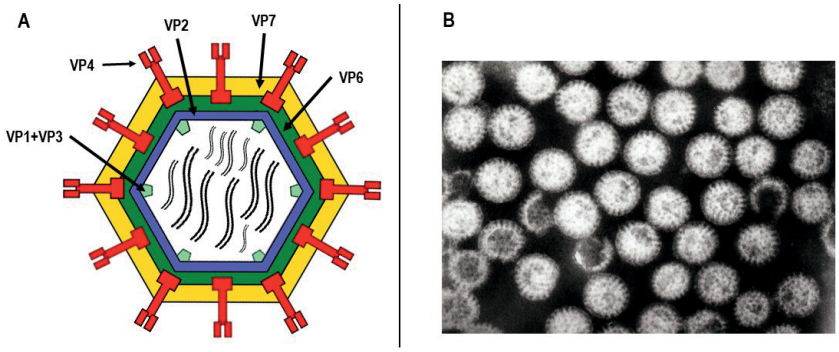

Figure 1. A) Schematic illustration of the structure of rotavirus. B) Multiple rotavirus particles detected by transmission electron microscope. Electron microscope picture: Dr Graham Beards under the Creative Commons Attribution 3.0 Unported License(40).

2.1.2 Classification and nomenclature

RVs belong to the genus *Rotavirus* of the *Reoviridae* family. RVs have been traditionally classified into groups (I or II) by serological methods based on the antigenic properties of VP6, the inner layer protein. More recently, classification has been made according to the amino acid sequence of the VP6. On the basis of our current knowledge, there are eight different serogroups (A to H) which infect different species. Serogroups A to C and H are known to infect humans. Group A RVs cause more than 90 % of all RV infections in humans.(41) Genetic reassortment is possible only between RVs of the same group(42).

RVs are further divided into genotypes, or G- and P-types, according to the nucleotide sequences of the outer layer proteins VP7 and VP4, respectively. G stands for "glycosylated" and P for "protease-sensitive", according to the main function of the protein. To date, at least 27 different G-types and 35 P-types have been identified.(43,44) Genotypes of the other nine RV group A genes have also been identified, and the schematics for representing the whole genome constellation of RV, VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, has been defined using the following abbreviations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where x is an Arabic number; indicating the genotype(44). Genotypes can be further divided into subgenotypic lineages on the basis of phylogenetics. Antigenic properties may differ greatly between the lineages of the same genotype (45).

2.1.3 Replication

RV infects mature enterocytes in the middle and the tip of the villi, and also the enteroendocrine cells in the small intestine(46,47). The RV replication cycle can be divided into five steps: attachment to the host cell, cell entry, transcription and translation, virus particle assembly and maturation, and release.

RV attachment to the host cell is a complex process that is still partly unknown. The process begins when intestinal protease-like trypsin cleaves VP4 into VP8* and VP5* particles. This causes VP5* to undergo conformational change which causes the protein to become more rigid and expose hydrophobic loops, both of which are important for cell entry(48,49). Binding is initiated by VP8*, which has been shown to interact with sialic acid on cellular glycans and histo-blood group antigens(50–52). After initial attachment by VP8*, several co-receptors – such as integrins and heat

shock protein 70 – also take part in the process and interact with VP5* and VP7 to mediate viral entry(50,53).

RV cell entry is strain-dependent as most strains enter using clathrin-mediated endocytosis, but other pathways have also been reported(49,54). As the RV-containing endosome enters the cell, the calcium level decreases, and the calcium-active VP7 layer is solubilized together with VP8* and VP5*. This increases the permeability of the endosomal membrane, and the transcriptionally active double-layered particles (DLP) are released into the cytoplasm.(55–58)

Release of DLPs activates the transcription of RV plus-strand RNA, which is facilitated by 12 transcription complexes (VP1+VP3) attached below the inner VP2 layer. Each complex transcribes only one dedicated genome segment. Transcribed RNAs are capped and extruded from the DLP into the cytoplasm through type I channels.(21,59) Antibodies against VP6 attach over these channels and inhibit intracellular translation(24). Plus-strand RNA serves as a template for the translation of viral proteins but also as a template for genomic dsRNA production(60). RV utilizes the host cell's protein synthesis machinery, and viral mRNAs compete with cellular mRNA for translation. NSP3 bound to viral mRNA enhances viral RNA translation while similarly interfering with cellular protein synthesis(36,61).

Newly translated viral proteins and plus-strand RNA accumulate into viroplasms, cytoplasmic inclusions formed by NSP2, NSP5 and intracellular lipid droplets(31,62–64). VP1 and VP3 form 11 polymerase complexes that bind to specific plus-strand RNA segments that each encode different proteins(65). Synthesis of dsRNA is activated when the complex attaches to the self-assembled VP2 layer. After completion of the VP2 layer, the middle VP6 layer self-assembles to form DLPs.(66,67)

Nascent DLPs are then budded through the endoplasmic reticulum for maturation. During the formation of the outer layer, DLPs are transiently enveloped. First, VP4 trimers are attached over the VP6 layer in interaction with NSP4, and finally VP7 trimers are layered on top, securing the VP4 trimers in place and completing the infectious triple-layered particle structure(66,68,69). Mature virions are then released from the cells by direct lysis or by the Golgi-independent transport system(70,71).

2.1.4 Antigenic epitopes and antibodies

The structure of RV contains several antigenic structures. To date, at least VP7, VP4, VP6 and NSP4 have been shown to induce a monoclonal antibody (mAb) response. The outward-facing side of the outer layer protein VP7 contains two antigenic epitope regions, 7-1 and 7-2, of which the first is divided into subregions 7-1a and 7-1b. Both, 7-1 and 7-2 have been shown to be bound with numerous different mAbs mainly in a serotype-specific manner.(18) Antibodies against VP7 have been shown to neutralize the virus by stabilizing the VP7 structure and blocking the uncoating of the VP7 capsid(18,72).

The trypsin-cleaved progeny of the intermediate-layer protein-sensitive VP4 protein, VP8* and VP5*, both contain several antigenic sites: VP8* four (8-1, 8-2, 8-3 and 8-4) and VP5* five (5-1, 5-2, 5-3, 5-4 and 5-5)(48,73,74). Anti-VP4 mAbs have been thought to neutralize and affect in a serotype-specific manner mainly against VP8*, hence inhibiting viral binding. However, Nair et al.(75) showed that anti-VP5* mAbs were also neutralizing, but the mechanism is yet unknown(76–78). The authors showed that VP5* mAbs generated broad heterotypic protection in humans that is not serotype-related(75).

Similar to the other two capsid proteins, the most abundant inner layer protein, VP6, has antigenic and immunogenic properties and two distinguished epitope regions(24,79). Anti-VP6 mAbs bind to the transcriptional pores on the surface of the VP6 layer, inhibiting transcription, which leads to intracellular neutralization, that is not shown in traditional *in vitro* neutralization studies(80,81).

Of the non-structural proteins, some reports have been published on the protection elicited by NSP4, the viral enterotoxin. In two mural studies, NSP4 antiserum showed protection from RV diarrhea when immunized before infection, and in the other study, it also halted the disease process when administered after the onset of diarrhea.(39,82) However, immunoglobulin (Ig) G levels against NSP4 have shown to be modest in humans questioning the role of NSP4(16,83).

2.2 Rotavirus disease

RVs are highly contagious due to three factors: Firstly, the infective dose of RV is low and only a few viral particles are needed for clinical infection(84). Secondly, the virus is transmitted through the fecal-oral route, and it is able to survive on different surfaces and media even for months. In addition, aerosol as a possible transmission

route has been speculated for more than 30 years without solid evidence.(85) Thirdly, a large number (up to 10^{10} particles per gram) of viruses are shed into stools already before the onset of symptoms, and shedding may continue for weeks after infection(86–88).

2.2.1 Clinical picture and pathophysiology

The clinical picture of RV infection in children is broad, ranging from asymptomatic infection to severe, life-threatening AGE. The infection usually begins with vomiting and fever that are accompanied 24 to 48 hours later with watery diarrhea that ranges from mild to severe. The combination of diarrhea, vomiting and fever may lead to severe dehydration, hyperchloremic acidosis and in the worst case to death.(89,90) RV incubation time ranges from one to two days and the illness itself is usually lasts 5-7 days(89,91).

Diarrhea

To date, the exact mechanism of RV-associated diarrhea is not fully understood, and it is likely that there are more than one mechanism causing. One suggested mechanism is secretory diarrhea caused by the viral enterotoxin NSP4 and enteric nervous system activation. The association of NSP4 with diarrhea was first detected in mice(39). Later studies have revealed that the binding of NSP4 to intestinal cells activates signaling system, which promotes calcium-dependent chloride channels(92,93). This leads to secretion of chloride ions into the gut lumen and to an increased osmotic gradient transporting water, resulting in secretory diarrhea. NSP4 also increases intracellular calcium levels, which increases serotonin release from the enteroendocrine cells; this in turn increases intestinal motility by activating the enteric nerves innervating the small intestine(94,95).

Another mechanism may be malabsorption in the small intestine due to enterocyte damage and death. Only a few biopsies from children with RVGE have been studied, but they have shown an irregularity of the mucosal cell lining with shortening and blunting of the villi together with an increase of inflammatory cells in the lamina propria(1,96,97). It is known that NSP4 has an effect on cell apoptosis but also causes changes in the tight junctions of the intestinal cell lining leading to increased permeability(98,99). This theory has been criticized, however, as oral rehydration therapy has shown great response in RVGE(53).

Vomiting

Release of serotonin due to NSP4 has been shown to activate afferent vagal nerves, a two-way pathway between the gut and the brain, causing stimulus in the vomiting center and producing vomiting and a sense of emesis during RV infection(47). Liquid flow through the gut has also been shown to be delayed during acute RV infection, also causing vomiting and nausea. Gastric emptying is also regulated by the vagal nerve circuitry, and therefore it is likely that the NSP4-serotonin pathway may cause decreased motility, but the relation to RV is still to be determined.(53,100)

Fever

Elevated levels of different cytokines functioning as endogenous pyrogens – including interleukin-6 and tumor necrosis factor-α – have been reported in the serum of feverish children with RVGE(101).

Extraintestinal spread

Even though the gut is the main organ system for RV replication, recent studies have shown that the spread of RV antigens into the serum (antigenemia) is common (in up to 90 % of children showing gastroenteritis symptoms) also in immunocompetent children, and it is associated with a more severe clinical picture(102,103). In some cases, antigenemia is also related to viremia, the presence of whole infective RV particles in the circulation(102). However, the clinical significance of viremia has remained unknown. In addition, there have been case reports of RNAemia; RV RNA has been detected in several different tissues, such as cerebrospinal fluid of children with meningitis(104), and in the liver and biliary tract tissue of children with biliary atresia(105).

Vesikari Score system

Several numeric scales have been developed to assess the severity of AGE and as a scientific tool to study vaccine impact. The most commonly used is the 20-point Vesikari score system(106). The grading of symptoms is shown in Table 1. The severity is scaled according to total points as follows: <11 points – mild, 11-15 points – moderate, ≥16 severe.

Table 1. The 20-point Vesikari scoring system.

Symptom or sign	Points		
	1	2	3
Duration of diarrhea (d)	1-4	5	≥6
Max no. diarrheal stools/24h	1-3	4-5	≥6
Duration of vomiting (d)	1	2	≥3
Max no. vomiting episodes/24h	1	2-4	≥5
Fever	37.1-38.4	38.5-38.9	≥39.0
Dehydration	-	1-5 %	≥6 %
Treatment	Polyclinical rehydration	Hospitalization	-

2.2.2 Laboratory detection of rotaviruses

As previously described, RV was first visualized using electron microscopy, which was highly specific for detecting morphologically unique RV but had a low throughput of samples and was expensive as a diagnostic tool(1,2,107). Electron microscopy remained as the gold standard for RV detection for a long time, despite having a relatively low sensitivity of around 80 %. Over time, many other methods have been used for RV detection, such as immune-electron microscopy, latex agglutination and immunofluorescence. In diagnostic use, different enzyme immunoassay derivatives, such as enzyme-linked immunosorbent assay (ELISA), based on the use of RV antibodies to detect the RV antigen, have superseded the previous methods due to a combination of high sensitivity, low-cost and speed.(108–110)

ELISA has remained as the robust diagnostic method par excellence. The development of the reverse transcription-polymerase chain reaction (RT-PCR) has replaced serotyping by genotyping, and the method has several advantages in comparison to ELISA. It is more sensitive, and in addition to the positive-negative result, all gene segments may be sequenced and possible changes in the sequences traced(111). However, RT-PCR as a procedure is time consuming and the therefore RV antigen-detecting ELISA continues to be the main assay in diagnostics. Recently, due to the latest developments in the real-time RT-PCR technique, PCR has also increased its potential for detecting RV and other viral causatives of AGE in one rapid multiplex assay(112). In research use, next generation sequencing methods have been established for efficient sequencing of the whole RV genome(113).

2.2.3 Immune response

Human immune response to RV infection is multifactorial and still in many parts unknown. It consists of innate and acquired components of which the first initiates the response while recognizing the virus and thereafter signals an acquired response to initiate. Main features of host immune response against RV are summerized in Table 2.

2.2.3.1 Innate immune response

The innate immune system is complex but agile in eliciting the initial defensive measures when encountering microorganisms or foreign substances. It consists of several different components, including recognizing molecules, the complement system, antigen-presenting cells, macrophages, monocytes and granulocytes. Epithelial cells are also part of the innate immune system, as they form a physical barrier against pathogens and excrete chemokines and cytokines, signaling to and recruiting other cells.(114) The response varies depending on the infected cell type and the infecting RV strain, and therefore the following is only a short description of the innate immune response to RV.

Table 2. Main characteristics of innate and acquired immunity against rotavirus infection.

	Innate immunity	**Acquired immunity**
Rotavirus recognition	Intracellular receptors binding on RV	Antigens presented by antigen presenting cells (macrophages, dendritic cells)
Method of action	▪ Interferon mediated pathways leading to "antiviral state" ▪ Activation of acquired immunity	**B cell response** ▪ Production of mAb (IgA, IgG and IgM) ▪ Long-term protection **T cell response** ▪ B cell activation (CD4+) ▪ Direct cell lysis (CD8+)
Related cells	Epithelial cells, dendritic cells	Dendritic cells, B and T cells
Rotavirus escape methods	Inhibitory effects by NSP1	Mutations of antigenic sites decreasing affinity of mAbs

After cell entry, RV is recognized by and bound to several receptors – such as melanoma differentiation-associated gene-5, retinoic acid-inducible gene-I, and toll-like receptors – eliciting host transcription factors, including INF regulatory factor 3 and nuclear transcription factor-*κ*B(115,116). These factors furthermore activate the expression of type I INFs and INF-stimulated genes, leading to abundant excretion of different INFs and INF-stimulated genes, mainly INF-α and INF-β, to neighboring cells(33,115). INF-α and INF-β then activate the Janus kinase-signal transducer and activator of transcription protein signaling pathway, leading to upregulation of numerous INF-stimulated genes and other immune effector cells, and to an "antiviral state". However, to date the specific mechanisms of only a few INF-stimulated genes have been discovered but the role of INFs as a signal mediator in RV infection was established early on.(33,117,118)

Dendritic cells are probably the most important in eliciting the immune response against RV, as they combine both innate and acquired immune systems(119). It has been reported that plasmacytoid dendritic cells are highly resistant against RV replication, unlike other cells; they secrete INF-α and INF-β in high amounts after recognizing the viral particle(120). In addition, plasmacytoid dendritic cells have been shown to elicit T cell independent B cell activation in RV infected mice also mediated by type I INFs and to play a role in the induction of mucosal IgA response(119,121).

The interferon antagonist protein NSP1 has an important role in inhibiting the innate immune response against RV(26). In the light of the current knowledge, the effects of NSP1 are targeted mainly against the production of IFN-β. It mediates proteasomal degradation of the host transcription factors – such as INF regulatory factors 3 and 7 – needed in the transcription of IFN-β and in the indirect inhibition of nuclear transcription factor-*κ*B, all leading to a decrease in the production of INF-β(122,123). NSP1 also affects on the later stages of the "antiviral state" by inhibiting phosphorylation of the signal transducer and activator of transcription-1(124).

2.2.3.2 Acquired immunity

Acquired or adaptive immunity is the second line of defense, and it is needed in case the innate immune system cannot eliminate the pathogen. The acquired immune system is based on recognition of a specific antigen, a part of an infectious organism, which elicits an immune response. It acts together with the innate immunity, as antigen-presenting cells such as macrophages and dendritic cells are needed for pathogen recognition and to elicit a reaction in the cells of the adaptive immune

system. The system consists of B and T cells, of which the first act against pathogens outside human cells and the latter against the pathogens that have infected or have been taken into host cells.(114)

B cell response

B cells mediate humoral immunity by synthesizing and excreting antigen-specific antibodies. They are classified into immature, mature, memory and plasma cells, each having specific function. When a naïve mature B cell captures an antigen and in a lymph node presents it to a CD4+ T cell – also known as the helper T cell –, the B cell begins to rapidly proliferate and interacts with follicular dendritic cells presenting the same antigen. B cells with the highest affinity against the presented antigen are selected while the rest are removed by apoptosis. After interacting with the helper T cell, these proliferated high affinity B cells change the type of Ig produced from IgM to either IgA, IgE or IgG. These cells then specialize into plasma or memory cells by an unknown mechanism. The function of plasma cells is to synthesize and excrete antigen-specific Igs into the bloodstream. Some of these cells, called long-lived plasma cells, migrate into the bone marrow and secrete IgG and IgA in large amounts, eliciting a long-lasting protection against the pathogen. Long-lived memory B cells, expressing IgA, IgE or IgG, also enable a rapid response against a subsequent encounter with the antigen by turning into Ig secreting plasma cells.(114)

Of the Ig isotypes, IgM, IgG and IgA are the most common and have been shown to be important in the immune response against RV. IgM is the first antibody to be produced in RV infection. It lacks viral neutralization qualities, but it forms an antigen-specific B cell receptor together with IgD on the surface of the B cells, which is needed for isotype switch. IgGs are usually located in the circulation or lymphoid tissue, and are long-lived, with an approximate half-life of 23 days. Their main functions include opsonizing the attached antibody for phagocytosis, antigen presentation for natural killer cells, and activation of complement. IgA can be divided into serum and secretory IgA. Secretory IgA is synthesized and secreted by plasma cells located in Peyer's patches in the lamina propria, a layer of connective tissue beneath the mucosal epithelium. The synthesized IgA is then transported to an extracellular fluid compartment such as the gastrointestinal or respiratory tract.(114)

During a first-time encounter, plasmacytoid dendritic cells recognize RV, causing a type I IFN response; this leads to the initiation of a T cell independent B cell response and to a notable expansion in the number of B cells(119,121,125). This in

turn results in early mucosal synthesis and the secretion of RV VP6 specific low-affinity IgM, accompanied days later by RV-specific IgA, which is later on supported by T cell dependent B cell activation and abundant IgA synthesis(125–128). According to studies in mice, RV antibody response is regionally distributed, as IgA is abundantly synthesized in intestinal tissue and only fractions can be measured in the circulation during the acute phase. Systemic antibody response is more likely to be mediated by circulating RV-specific IgM and IgG.(129–132) However, the role of antibodies in the clearance of infection is not clear, as Franco et al.(133) showed that B cell deficient mice cleared the infection similar to controls.

Maternal antibodies

Anti-RV IgG is transferred transplacentally from the mother to the fetus while IgA is transferred to the newborn via the colostrum and breastmilk(134). The serum IgG levels of infants are similar to those of mothers, providing the newborn protection during the first months of life, after which the IgG level begins to decline until a further increase due to natural RV infection or vaccination.(135,136). Orally obtained IgA remains in the gut, providing temporal local protection as long as lactation continues(135). Both IgA and IgG levels in breastmilk are higher in low- than in high-income settings and could cause lower vaccine uptake and thereby be one explanation for the poorer vaccine real-life effectiveness seen in low income setting(137,138).

T cell response

T cells are divided into two main subgroups, CD4+ and CD8+, according to their surface co-receptor molecules. Naïve T cells are activated by an interaction with an antigen-presenting cell, such as a macrophage or dendritic cell, leading to proliferation, differentiation or survival. As earlier mentioned, CD4+ T cells are needed in B cell activation, but they also secrete several cytokines affecting numerous cells of both innate and acquired immunity. In the case of RV infection, subtype T_H1 CD4+ T cells produce IFN-γ and interleukin-2, which increases the activation of macrophages, CD8+ T cells and natural killer cells. CD8+ T cells are activated principally in the same manner as CD4+ cells, but in some cases, for an unknown reason, CD4+ cells are needed in the process. Their main function is to kill infected cells via two pathways, of which the major route increases the target cell's permeability by adding pores to the cell membrane; the other pathway causes

apoptosis by signaling pathway activation. After elimination of the T cell response, a small proportion of CD4+ and CD8+ T cells transform into memory cells, which enable a quicker and more efficient response in comparison to the initial response when the same pathogen is met again.(114)

The majority of studies on cell-mediated immune response against RV have been conducted in mice, and therefore the results cannot be directly extrapolated to humans. In murine models, CD4+ T cells have been shown to be crucial for eliciting B cell-mediated antibody response against RV and especially for long-term protection against subsequent infections(139,140). CD8+ T cells have been associated with RV clearance, but they also take part in short-term protection(140,141). It has been shown that CD8+ T cells are not absolutely necessary for clearance of RV, but their absence causes a delay in clearance(133,140,142,143). A lack of CD4+ T cells or both,CD4+ and CD8+ T cells, on the other hand, led to prolonged infection at a low level, showing T cell independent B cell activity controlling but not resolving the infection(142).

2.2.4 Long-term protection and correlates of protection

Cellular mechanisms of long-term RV immunity

Both B and T cells probably have an effect on long-term immunity. According to a murine study, a total lack of B cells leads to recurring RV infections, suggesting that antibodies are vital for the formation of long-term immunity, while the role of T cells is not as clear(133). Other murine studies have shown indications of the involvement of effector – but not memory – CD8+ T cells on short-term protection(141,144). CD4+ T cells are also involved in development of protection after parenteral inoculation with non-live VP6 particles(145).

As acute-phase antibody secreting plasma cells (ASCs) are rather short-lived, long-term protection is based on memory B cells. Yuan et al.(132) studied the locational distribution of plasma and memory cells in gnotobiotic pigs after oral inoculation with virulent and attenuated human RV strain and challenged the pigs with virulent strain. Inoculation with the virulent strain mimicking response to a natural RV infection; it elicited a high response in IgA ASCs and a moderate response in IgG ASCs and IgG memory B cells of intestinal lamina propria. Whereas vaccination with the attenuated RV strain – similar to current RV vaccination in humans – showed no ASC response, instead there was a high IgG memory cell

response in the spleen. After the challenge with the virulent RV strain, the responses between the groups were different. Pigs with the initially virulent infection showed only a moderate IgA ASC response, while pigs inoculated with the attenuated RV elicited an IgA and IgG ASC response in the lymphoid tissue of lamina propria. Memory B cells responses also differed, as pigs originally inoculated with the virulent strain showed a broad IgG-related response in the intestinal lymphatic tissue, spleen and peripheral blood, whereas in the attenuated group, the response was moderate, IgM- and IgG-related and only seen in the peripheral blood.(132)

Studies on memory T cell function in long-term protection are still underway. Offit et al.(146) measured increased lymphoproliferative activity in children up to eight months after RV infection. However, the increase began not until after the acute phase of primary infection. By contrast, Mäkelä et al.(147) did not find a similar persistence as the responses declined soon after RV infection, including also secondary infection. The authors used healthy adults as controls and found higher activity in asymptomatic adults than in infected children. A similar finding was made by Jaimes et al.(148), and both of the authors speculated that as RV-specific memory T cells express a gut-associated homing receptor, the number of the cells circulating in the peripheral blood is low but eventually increases to a significant level after an increasing number of reinfections(147,149).

Clinical correlates of protection

One of the first clinical reports on protection against natural RV infection was by Chiba et al.(150), who studied three consecutive RV epidemic seasons in an orphanage in Japan. The children had increasing serum antibody levels after consecutive infections, and in some cases, the second or third infection became asymptomatic suggesting that recurring infections could provide protection against following infections(150). Velázquez et al.(151) followed RV infections in a cohort of Mexican children for the first two years of life. In that study, the primary RV infection was the most severe while the following infections appeared with milder symptoms and eventually became subclinical. In fact, after two infections, protection against moderate to severe disease was calculated to be 100 %(151).

While in the clinical studies, protection by natural RV infection seems evident and inevitable with recurrent infections eventually leading to protection against further symptomatic infections, from the immunological perspective the case is not as unambiguous. The role of different Igs and antigenic structures has varied greatly between studies. In adults and children, some studies have shown that clinical

protection correlated with serum IgG(152,153), others have shown that serum IgA(154) was the determinant of protection, while in some studies(155,156) both of the presented Igs elicited protection. In early adult challenge studies, Kapikian et al.(157) and Green et al.(158) showed that clinical protection correlated with high serum neutralizing mAb titers against VP7 or VP4. Later studies in children have found a similar correlation(150,155). However, no such correlation was found in two studies by Ward et al.(152,159).

During the course of time and numerous clinical vaccine studies, the serum IgA level against VP6 has been shown to be a good – but not optimal – clinical correlate of protection, especially against severe RV disease, as measured in the licensure studies of the two current vaccines, RotaTeq and Rotarix(154–156,160,161). The protein is highly antigenic, as shown by Svensson et al.(15) and Johansen et al.(162), who studied serum samples obtained from children with radioimmunoprecipitation assays. In these studies, VP6 elicited a noticeably stronger serum antibody (IgG and IgA) response in comparison to other RV components, including VP7 and VP4(15,162). Murine studies have shown protection after systemic exposure to anti-VP6 mAbs and intranasal immunization with VP6, supporting the role of VP6 IgA as a mediator and correlate of protection(81,145,163). Despite being a good measure of clinical protection against wild-type RV infection, IgA is not needed for protection in murine model, unlike IgG(164).

Protection after natural RV infection has been shown to be highly homotypic, but the infection also elicits antibodies against other strains(150,165). Yuan et al.(166) suggested that protection against natural RV infection is mainly strain-specific (homotypic) and correlates especially with IgG against VP7 and VP4 but heterotypic protection was also detected(166). In a Mexican follow-up study by Velazquez et al.(151), only 2 out of 22 paired infections were caused by the same genotype, suggesting strain-specific protection. On the other hand, Chiba et al.(150) measured high antibody titers against the causative strain but increases in titers against other strains as well. Ultimately, the most conclusive evidence on behalf of heterotypic protection is the high efficacy and real-life effectiveness of the single human RV strain G1P[8] vaccine(161,167–169).

Vaccine elicited immunity

Patel et al.(170) analyzed clinical vaccine studies conducted on both of the currently used vaccines, Rotarix and RotaTeq, and found an inverse relation between measured serum IgA levels and childhood mortality in children aged under five years

of age – a higher post-vaccination titer resulted in lower mortality. Serum IgA levels also seemed to predict vaccine efficacy well for both vaccines. Another analysis made solely on Rotarix pre-licensure data found evidence of a relation between serum IgA seroconversion and efficacy(171). In fact, Patel et al.(170) made a similar observation regarding Rotarix, but in that material, seroconversion did not correlate with vaccine efficacy in children who received RotaTeq vaccine. Even though the role of serum IgA has shown some evidence as a marker for protection, the findings contain inconsistencies, signifying that natural as well as vaccine-induced protection against RV is multifactorial. It is as well likely that the immunity provided by natural RV infection and vaccination differ.

2.3 Epidemiology of rotavirus

2.3.1 Burden of disease

In 2016, it was estimated that RVGE causes approximately 230,000 deaths in all age groups globally, of which 130,000 occur in children under five years of age(7). Before the licensure and introduction of RV vaccinations, the annual death toll was more than 450,000 only in children younger than five years(5). Geographically, mortality is greatly unbalanced, as most deaths are seen in developing countries with limited healthcare resources, a lack of clean water and childhood malnutrition. While death due to RV is rare in industrialized countries, RV is still a common cause for hospitalization, thus inducing an economic burden(9,172–174).

Rotavirus seasonality

No certain pattern has been identified to explain differences in the seasonality of RV disease between different countries. For a long time, it was thought that climate would explain why clear high activity seasons are present in temperate zones while the disease is seen throughout the year in the tropics(175,176). However, more recent analyses have shown that although the weather might have an effect on RV circulation, the climate does not explain changes in RV activity, which rather is a sum of several factors. In fact, income level has been shown to be a better predictor of annual fluctuations compared to climate zones(177). Furthermore, recent

computer models have shown that changes in birth-rate may have an effect on RV epidemics, as epidemics occur earlier during the years with a high birth-rate(178).

2.3.2 Circulating rotavirus strains

Surveillance of circulating RV strains began already in the 1970s. The primary goal of the surveillance was to gain better knowledge of the pathogen, then to collect strain-specific data for vaccine development and finally to study the effects of RV vaccines on the circulating strains. During this more than 40-year period of follow-up, despite natural fluctuations in the circulating strains, four main genotypes remained common: G1P[8], G2P[4], G3P[8] and G4P[8]. Globally, these genotypes have caused approximately 90 % of all RV cases, G1 being the most common serotype(179,180)

A large review by Santos et al.(180) covering the pre-vaccination era from the 1970s to 2003 reported geographical differences in the genotype distribution, as G1P[8] was most common in Australia, Europe and North America, while in Africa, Asia and South America other genotypes, such as G3, G4 and G8 were more common during that 30-year period. In addition, the number of different uncommon combinations of VP7 and VP4 was far higher in these areas, forming nearly a third of the cases (Fig. 2). Of the VP4 types, P[6] was practically seen only in Africa, where it was the second most common P-type after P[8], while in other regions, P[8] and P[4] were seen in more than 90 % of the cases.(180)

Before the introduction of RV vaccines, it was speculated that the strains contained in the vaccine would be suppressed and new strains would emerge as a cause of mutations and reassortment caused by the vaccines. However, already at the end of the 1990s, before the licensure of RV vaccines, the G9P[6] strain emerged globally at a common level and through reassortment with a P[8] strain, the formed strain G9P[8] became the fifth main RV strain(181–186). A few years later, in the mid to late of 2000s, another novel genotype, G12P[8], was detected in several countries and the prevalence soon rose to the common level, becoming the sixth main RV strain to date(187–190). Phylogenetic analyses have shown that the strain originated from porcine G12 that has reassorted with the human G1P[8] strain(191,192). It was reported early on that these novel strains were more capable of causing severe clinical disease, but in total the results are controversial, as there are reports showing similar or milder severity in comparison to the "original" strains such as G1(188,193–197).

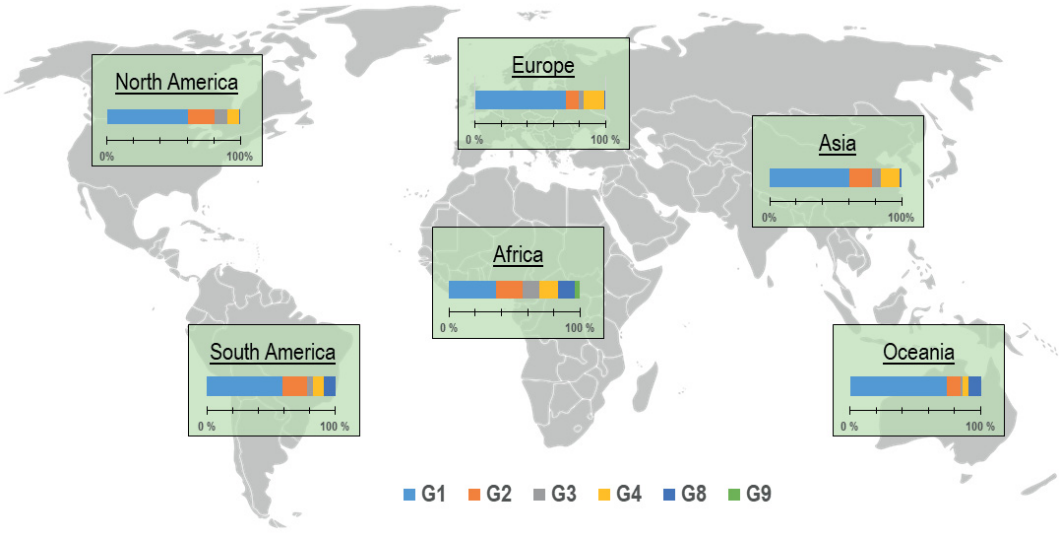

Figure 2. Geographical distribution of the most common rotavirus G-types before rotavirus vaccine introductions(180). World map by user: Vardion, modified under the Creative Commons Attribution 3.0 Unported License(40).

In addition to geographical differences, circulating strains have a natural tendency to fluctuate year-to-year and even during a single season. In a large study analyzing RV genotypes and the occurrence during and outside the high season in Europe, it was noted that G1P[8] was dominant during the high seasons, but this predominance declined during the off seasons and other genotypes became more frequent(198). Analysis of epidemiological genotype data has suggested that the period of predominance of one strain ranges from 3 to 11 years, and in the case of G1, the cycle is on average longer, from 5 to 11 years(199). In fact, Pitzer et al.(199) speculated that a possible explanation for the cyclic nature of the predominant strain might lie in homo- and heterotypic immunity. The fluctuation of RV genotypes is the sum of several factors on the individual and population level. The accumulation of children with a strong homotypic immune response against the predominant strain may predispose to infection by heterotypic strain, which eventually may lead to ousting of the predominant strain.(199)

Finland

The earliest data describing RV epidemiology in Finland dates back to the 1980s, when over four seasons of follow-up (1986-1990), G1 was the most common genotype. However, natural fluctuation of RV genotypes was apparent, as G4 was

by far more common during the 1988-89 season.(200) A similar predominance of G1 continued also in the 1990s and up to two seasons (2006-2008) before the vaccine introduction in 2009(201,202). No major changes in the circulating genotypes were seen during the first two seasons (2009-2011) after the introduction of the RotaTeq vaccine at the beginning of September 2009. Although G4 was indeed more common during the first season, G1 retained its predominance during the latter season.(203) Another two season (2012-14) follow-up was conducted by Hemming-Harlo et al.(8) showing a low RV presence overall. The low total number (40 cases) of wild-type RV cases detected during those seasons signaled the high effectiveness of the vaccine. Genotype distribution did not offer any surprises as G4P[8] (11 cases) was more common in total compared to G1P[8] (10 cases). G12P[8], a novel genotype, was observed for the first time in Finland during the 2012-13 season.(8)

2.3.3 Rotavirus in adults

RV was noted also as a common pathogen for adults in the 1970s, but due to the outbreak occurrence and the milder or even asymptomatic clinical picture, RV has been viewed as a disease of children(204–206). Therefore the occurrence of RV in adults has remained partly less known, but it has been estimated that in the elderly population aged 70 and above, RV causes approximately 58,000 deaths annually(7). In addition, pathogen testing is not standard of care in adults with periodic diarrhea in most countries. The notion of previous RV infections being protective against the development of severe symptoms in recurring infections in children probably expands also into adults and the elderly(156).

The incubation period of RV in adults ranges from two to six days; this is longer than in children, whereas the duration of illness itself is usually shorter, one to four days, in adults. The clinical picture varies greatly(157). The main symptoms are the same as in children, but dehydration is more seldom in adults. The development of a more severe clinical picture or prolonged diarrhea is usually related to immunocompromising or other co-morbidities such as malignancy or diabetes(207–209). In the US, approximately 30 % of the cases are associated with lowered immune response(210).

RV disease in adults is mostly endemic and cases are seen throughout the year, whereas in children there is a clear high season during the winter months(176). The prevalence of disease in adults ranges from the estimated 3 to 18 % in the US, to

14 % in Japan and even up to 63 % seen in Mexico during the winter months(176,211,212). However, most of the studies evaluating RV prevalence in adults were conducted before licensure of RV vaccines, and therefore the numbers are probably overestimates of current situation, as a 50 % decrease in the RV cases of adults was seen after the initiation of pediatric RV vaccinations in the US(210). A similar decrease in all cause AGE was reported as well in all adult age groups in the UK, but to a lesser extent(174,213). Even though the decline of the burden of RV disease in adults after the vaccine introduction suggests transmission from children to adults, there are studies were such an association has not been found(207,214).

The most common genotypes are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8], similar to those found in children(209,215–218). Several reports of outbreaks in nursing and retirement homes have shown that RV can also potentially cause a local epidemic in a closed community of adults and the elderly even without potential disease transmission from children. These outbreaks have mainly been caused by G2P[4], but there are reports of G4P[8], G8P[8] and G12P[8] being the causative genotype.(206,219–225) RV has also been shown to be a noticeable viral pathogen in travelers' diarrhea causing approximately 10 % of all cases, especially in Africa and Latin America(226,227). Travel may also enhance transmission and the spread of novel genotypes around the globe(209).

2.4 Rotavirus vaccines

2.4.1 Development of rotavirus vaccines

The significant RV-related morbidity and mortality created a drive to develop a vaccine against the virus. On the basis of studies involving adult volunteers, it was assumed that fecal IgA levels would be the best correlate for host response and therefore an oral live vaccine would be the best option.(228) It was also noted in a small subset of children that sequential RV infections were caused by different RV serotypes signaling immunological memory against certain strains(229). However, as the knowledge about the virus and immunity was slender, the approach used in vaccine development was Jennerian, taking advantage of cross-reactivity between similar group antigens of human and animal RVs also known as serogroups. Animal RVs were preferentially selected as they belonged to a similar serogroup (VP6) (RIT4237, RRV) as circulating human RVs G1 and G2 and/or because they had

been shown to cause asymptomatic or mild infection in humans (WC3) and because they grew well in cell cultures. Human RV strains could not be used until it was learned that their growth in cell culture could be enhanced by trypsin. Following paragraphs discuss the most important vaccine candidates, currently used and some of the future RV vaccines still underdevelopment.

Summary of the vaccines discussed during the following paragraphs is presented in Table 3.

Table 3. Summary of past, current and future rotavirus vaccines and vaccine candidates.

Past vaccine candidates	RV strain	Characteristics
Animal		
RIT4237	Bovine G6P[1]	Modest VE, well-tolerated
WC3	Bovine G6P[5]	Low VE, well-tolerated. Further development to RotaTeq
RRV	Rhesus monkey G3P[5]	High VE (homotypic only), post vaccination fever reactions
Animal-human reassortant		
RRV-TV, Rotashield	Human G1, G2, G4 VP7 on rhesus G3P[5] backbone	High VE, well-tolerated. Withdrawn due to increased risk for intussusception
Human-bovine(UK) reassortant	Human G1, G2, G4 VP7 on bovine UK strain	High VE, well-tolerated
Human		
M37	G1P[6]	Poor VE, well-tolerated
89-12	G1P[8]	High VE, well- tolerated. Further development to Rotarix
Current main vaccines		
Rotarix	Same G1P[8] as 89-12	High VE (high income countries), well-tolerated. Globally most used RV vaccine.
RotaTeq	Human G1, G2, G3, G4 and P[8] on bovine G6P[5] backbone	High VE (high income countries), well-tolerated. Used in Finland since 2009.
Other currently used vaccines		
RotaSiil	Human-bovine reassortant G1-G4 and G9	VE similar to seen by the main vaccines in a low income setting. Both licensed and used in India.
Rotavac	Human G9P[11]	
Rotavin-M1	Human G1P[8]	Developed and used in Vietnam
Lamb RV	Lamb G10P[12]	Developed and used in China.
Under development		
RV3-BB	Naturally attenuated G3P[6]	Decent VE, currently in clinical studies
Parenteral P2-VP8-P[8]	VP8* P[8] fused to tetanux toxin	Clinical studies on humans
Rota- and norovirus combination vaccine	Recombinant VP6	In mural studies

VE = vaccine efficacy

2.4.1.1 Animal rotavirus vaccines

RIT4237

RIT4237, the first oral RV vaccine candidate that reached clinical trials in children, was based on a cell culture-attenuated (154 passages) bovine RV G6P[1] strain that was originally isolated from calves in Nebraska(230,231). The G6P[1] strain shared the same VP6 (subgroup 1) with the other most common human RVs, and it also showed cross-reactivity against other subgroups (2 and 3)(230). RIT4237 showed promising results in the first efficacy trials conducted in Finland in 1983, showing 50 % and 88 % protection against any and severe RV disease, respectively, with only one dose(230). However, in studies done in developing countries, the efficacy was seemingly lower: In Peru 40 % and 75 %, respectively, and in Gambia with only 33 % efficacy against RV disease of any severity(232,233). The vaccine was withdrawn from further development due to the modest efficacy in developing countries.

WC3

The vaccine based on cell culture-attenuated bovine RV WC3 strain G6P[5]. In the early studies, WC3 showed better serological results with lower inoculum than RIT4237, producing less adverse effects, and therefore it was considered as a potential vaccine candidate.(234) However, the vaccine showed low efficacy against human RVs in clinical studies and further development was halted(235,236).

RRV

Rhesus RV, *RRV,* vaccine strain (G3P[5]) originated from stools of a rhesus monkey(237). The strain was attenuated and adapted to vaccine use by cell culture. The strain was selected because it was not reported to infect humans and induced neutralizing antibodies against serotype 3(238). In clinical studies, RRV was shown to be highly efficacious (80 %) against homologous RV strains, whereas efficacy against heterologous strains was only 48 %(239–241). The vaccine was more antigenic compared to RIT4327, but it showed higher reactogenicity, causing fever reactions 3-4 days post vaccination(239,242,243). The lack of heterotypic protection and the increased reactogenicity led to the end of development of a pure RRV vaccine.

2.4.1.2 Animal-human reassortant vaccines

RRV-TV, Rotashield

The observation of natural reassortment of genes between two RV strains infecting the same cell culture inspired the creation of a reassortant vaccine, which combined lower reactogenicity of animal RV and the broad serotype protection of human RV(244). This resulted in RRV-TV, the rhesus-human reassortant tetravalent vaccine, which was later licensed as *RotaShield* (Wyeth). The vaccine contained human G1, G2 and G4 VP7 proteins on a rhesus G3P[5] RV backbone.(245,246) The used vaccine dose was lower than in previous RRV studies, which resulted in fewer fever reactions following vaccination(247). In the largest clinical studies conducted in the US and Finland, the vaccine showed to be highly efficacious, especially against severe RV disease (80-91 %) while the efficacy against all-severity RVGE varied from 49 to 66 %(248,249). However, in the Latin American countries of Venezuela, Peru and Brazil, the efficacy was lower, and ranged from 26 to 48 % against any RV disease and from 30 to 88 % against severe RVGE(250–252). The vaccine was administered in three doses at the ages of two, four and six months.

RRV-TV was licensed in the US in 1998, and was in use up to 1999, when it was withdrawn due to an association with an increased risk for IS after vaccination(6). Nonetheless, in terms of real-life efficacy, RRV-TV was highly efficacious, as seen in a retrospective case-control study conducted by Staat et al.(253), where the real-life efficacy of the full RRV-TV regimen against hospitalization was 100 % and even a single dose provided 89 % efficacy against RV-associated hospitalization. More recently, the vaccine has been studied in Ghana, where it was given in neonatal setting in a two-dose schedule to minimize risk of IS(254).

Human-bovine (UK) reassortant vaccine

Concomitantly with the development of RRV-TV a similar tetravalent RV vaccine based on a bovine UK strain was studied. The vaccine showed similar efficacy as RRV-TV and was well tolerated(255). However, as RRV-TV was withdrawn due to intussusception, the development of the human-bovine RV vaccine was also discontinued by Wyeth.

2.4.1.3 Human rotavirus vaccines

M37

The M37 (G1P[6]) strain was obtained from the stools of a neonate with an asymptomatic RV infection in Caracas, Venezuela in 1982(256). The strain was attenuated by propagation in African green monkey cells for 21 passages. The vaccine was well-tolerated but only moderately immunogenic, and it showed poor heterologous seroresponses.(257,258) In an efficacy trial conducted in Finland, the M37 vaccine showed no clinical protection against RV disease(259).

89-12

The human RV vaccine candidate based on the strain 89-12 (G1P[8]) was isolated from the stools of a child with RVGE in Cincinnati, USA, during the 1988-1989 RV season(236). The strain induced a broad cross-reactive neutralizing antibody response and the infection caused by the strain seemed to protect against subsequent RV infection(260,261). The strain was attenuated by a total of 33 passages in two African green monkey kidney cell lines. The vaccine was chosen to be given in two doses. Safety and immunogenicity studies showed that the vaccine was well tolerated and immunogenic.(262) The initial efficacy trial, conducted in the US, showed high (89 %) efficacy against RVGE of any severity and 78 % efficacy against severe RV disease(263). In a two-year follow-up, the efficacy remained high at 76 % against any severity RVGE and 84 % against severe disease(264). The strain was further developed, and the final vaccine became known as Rotarix.

2.4.2 Current rotavirus vaccines

2.4.2.1 Human rotavirus vaccine, Rotarix

The most used RV vaccine globally, Rotarix (GSK, Rixensart, Belgium), originates from the 89-12 strain. The strain was cloned by plaque purification and further propagated for another 10 passages in Vero cells to develop the RIX4414 strain used in the vaccine. In the pilot safety and immunogenicity study, RIX4414 showed to be highly immunogenic, similar to 89-12, and was tolerated even better than the predecessor strain.(265) In an efficacy study conducted in Finland, RIX4414 showed

72 % efficacy against RV disease of any severity and 85 % efficacy against severe RVGE after the administration of two doses(266). Other studies from Singapore and Latin America showed the vaccine to be well tolerated, that the vaccine efficacy was high (up to 86 % against severe RVGE), and that the vaccine was also highly efficacious (up to 83 %) against non-G1 strains (267–269). A large European efficacy study confirmed the high efficacy against severe (up to 90 %) and any severity (up to 87 %) RV disease(270).

A large phase 3 safety and efficacy trial was conducted in 11 Latin American countries and Finland, and contained 63,000 infants who received two doses of the Rotarix vaccine. The vaccine showed 85 % efficacy against severe RVGE and an efficacy of at least 87 % against genotypes G1P[8], G3P[8], G4P[8] and G9P[8]. However, against the completely heterologous strain G2P[4], the efficacy was only 41 %. Overall, the vaccine was assessed as well tolerated and safe. IS was detected in six children who had received the vaccine and in 16 placebo recipients, indicating a low risk of IS.(161)

2.4.2.2 Human-bovine reassortant vaccine, RotaTeq

The human-bovine reassortant vaccine, RotaTeq (Merck, Kenilworth, NJ, US), was developed in similar manner as RRV-TV, reassorting four human VP7 genes (G1, G2, G3 and G4) and human VP4 gene P[8] into a bovine WC3 G6P[5] strain backbone. Human RVs were isolated from the RV positive stools samples of infants in the Children's Hospital Philadelphia.(271) The WC3-based vaccine, as discussed earlier, was found to show poor protection against human RVs, but it was highly immunogenic and well tolerated, and therefore it was assessed as a good template for further vaccine development when reassorted with more immunopotent surface proteins from human RVs. The monovalent proof of concept vaccine contained only G1 VP7, but it showed high efficacy, from 64 to 100 %(272,273). Other reassortants containing three other VP7s and VP4 were added to the vaccine to broaden the spectrum of protection to cover all major wild-type RV strains(271). Bivalent (G1+G2) and quadrivalent (G1-G3 and P[8], and G1-G4]) compositions were tested showing similar or even higher efficacy(274–276).

RotaTeq went through a large efficacy and safety trial named the Rotavirus Efficacy and Safety Trial (REST). The placebo-controlled randomized trial included over 70,000 participants and was conducted in 11 countries including the US and Finland. The vaccine showed 74 % efficacy against RVGE of any severity and 98 % efficacy against severe disease.(160) In the original study, the efficacy was shown

against genotypes G1-G4 contained in the vaccine, but the Finnish Extension Study showed that the RotaTeq vaccine was likewise highly (92 %) efficacious against G9P[8], a partly heterotypic strain not contained in the vaccine(277). The REST study showed major reductions in RV-related healthcare visits, as in the follow-up up to two years after vaccination, a 96 % reduction in hospitalizations and a 94 % reduction in emergency department visits was noted. In the REST material, the number of IS cases was lower in the vaccine recipients compared to the recipients of the placebo which was an important finding after the withdrawal of RotaShield due to IS.(160)

RotaTeq was added to the Finnish National Immunization Program (NIP) in the beginning of September 2009. The vaccine is given as a three-dose regimen at the ages of two, three and five months with minor national variance. First-dose vaccination coverage has been over 90 % since the introduction(9).

2.4.2.3 Other rotavirus vaccines and vaccines under development

Several other RV vaccines have been developed and taken into use, mainly in Asia. Two of these vaccines, RotaSiil (human-bovine reassortants G1-G4 and G9) and Rotavac (G9P[11]) are currently used in India and have also been prequalified by the World Health Organization(278–280). The efficacy of both of these vaccines has been 35 % against any severity RVGE and 55 to 67 % against severe RVGE(281,282). This is somewhat similar to the efficacy shown by Rotarix and RotaTeq in developing countries(283,284). Other vaccines know to be in use are the single attenuated human RV G1P[8] vaccine Rotavin-M1 in Vietnam and the lamb RV (G10P[12]) vaccine in China. The published efficacy data for the latter two vaccines is lean(285,286).

Of the RV vaccines still under development, RV3-BB is based on the naturally attenuated human RV strain G3P[6]. The strain has been shown to cause asymptomatic infection in newborns and similarly to provide protection against severe RV infection.(287) A more recent efficacy study showed a 75 % efficacy using an infant administration schedule (8, 14 and 18 weeks) and 51 % efficacy in the neonatal schedule (0-5 d, 8 and 14 weeks)(288).

Third generation rotavirus vaccines

Despite the second-generation live oral RV vaccines being shown to be highly efficacious in developed countries, in developing areas the effectiveness of the

vaccines has remained low to moderate despite high vaccine coverage(289). As basic studies have tried to explain the difference seen in effectiveness, vaccine development of third-generation RV vaccines has focused on non-live RV particles usually administered parenterally or intracutaneously. With no RV replication in the gut, these vaccines would have the potential advantage of a lower risk of IS. It would also be possible to combine these subunit vaccines together with other childhood vaccines, which could improve vaccine coverage.

Approaches in the field are numerous, but the parenteral P2-VP8-P[8] subunit vaccine was the first third-generation RV vaccine to advance to clinical trials(290,291). The vaccine is based on the tetanus toxin P2 epitope fused truncated VP8* protein, which contains most of the neutralizing epitopes of the whole VP4 protein(292). The vaccine has been shown to be immunogenic and well tolerated in children(291). No efficacy data has yet been published. Further studies are currently ongoing on a trivalent (P[4], P[6] and P[8]) composition(291). In addition, a combination subunit vaccine against RV and norovirus is currently under development. Recombinant VP6 protein, which has been produced in the baculovirus system in insect cells, functions as the RV subunit inducing neutralizing antibodies and also as an adjuvant for the norovirus component of the vaccine(293).

2.4.3 Vaccination of adults

Even though adults have usually been the first subjects in RV vaccine clinical trials, the vaccine safety, immunogenicity and efficacy in this population has not been well determined. In 2014, Lawrence et al.(294) conducted a clinical trial assessing the safety and immunogenicity of the RotaTeq vaccine in 66 elderly people (aged 65-80 years). Two thirds received the three-dose RotaTeq vaccine regimen and one third formed the placebo group. The vaccine was determined to be safe and well tolerated in this age group with no reports of severe adverse events. The study reported for the first time RV serology of the elderly showing relatively high rotavirus antibody levels reflecting to previous infections. Despite of the antibody levels, increase of serum neutralizing antibody titers against RV was detected, and roughly a third of the recipients had a 3-fold rise after three vaccine doses. The results indicated that despite previous RV exposure and waning immunity, the RV vaccine may have potential in this age cohort.(294) However, no further studies have been conducted.

2.4.4 Effects of universal rotavirus vaccinations

Already in 2009, the World Health Organization recommended the inclusion of the RV vaccination into vaccination program of every country. To date, RV vaccines have been introduced in 98 countries and the number is increasing. The proportion of countries using RV vaccine varies geographically. The highest proportion of countries using RV vaccines are in Africa, where the burden of disease and mortality is highest as well, whereas in developed regions such as Europe, the proportion is lower.(295)

2.4.4.1 Real-life vaccine effects and effectiveness of current rotavirus vaccines

Since the introduction of the RV vaccines, the number of annual RV related deaths in children under five years of age has decreased from 450,000 to 130,000(5,7). According to a recent study by Troeger et al.(296), during the last 26 years, from 1990 to 2016, RV-associated mortality has decreased by 48 % in children under five years of age. It has been estimated that globally RV vaccines cover only 28 % of children aged under five, but they still averted 28,000 RV related deaths in 2016(296).

In Finland, high-coverage RV inoculations have resulted in an over 90 % reduction in RV-related hospitalizations and outpatient visits(9,297). Mass vaccination has also decreased all-cause AGE hospitalizations by 69 %.(9) In the US, another country mainly using RotaTeq, RV-related hospitalizations of children under the age of five years have decreased by 84 %(298,299). Similar reports have been published also from other high vaccine coverage countries, such as Belgium and England who both use Rotarix with a 85 % and 77 % reduction, respectively, in RV hospitalizations in comparison to the pre-vaccine period(213,300).

Both the Rotarix and RotaTeq have shown high real-life vaccine efficacy (VE) in post-licensure clinical studies; however, a clear RV mortality-related gradient is present when comparing VE between different regions(301). In high income countries with low RV mortality, such as Belgium (Rotarix), Finland (RotaTeq) and the US (both, mainly RotaTeq), post-licensure studies have reported real-life vaccine effectiveness against hospitalization ranging from 86 to 94 % for RotaTeq(297,298,302,303) and from 83 to 90 % for Rotarix(168,302). In pre-licensure studies, the VE of Rotarix against G2P[4] was significantly lower (41 %) than against other genotypes. However, in more recent clinical studies conducted in Belgium and Brazil, VE against G2P[4] ranged from 77 to 85 %(161,168,169).

In countries with moderate to high RV mortality, the effectiveness of current RV vaccines has been shown to be considerably lower. A consensus VE calculated by Jonesteller et al.(301) was 49 % for RotaTeq and from 58 to 66 % for Rotarix. While the RV vaccine related reduction in hospitalizations and emergency department visits of children aged under five years has been shown to be on average 71 % in low-mortality settings, in moderate and high mortality regions the reduction was lower, approximately 60 %. In children in their first year of life, the difference in median reduction between low (80 %) and high (46 %) mortality settings was even higher. Interestingly, there was no difference in reduction between low (80 %) and moderate (78 %) mortality areas.(289)

The difference between high and low income countries cannot be explained by circulating genotypes, as even though the number of strains is more diverse and they contain also the P[6] strains uncommon in high income settings and absent from the vaccines, the VE is similarly low also against the common strains such as G1(304). To date, multiple affecting factors have been proposed, including malnutrition (especially vitamin A and zinc deficiency), a higher proportion of antiviral glycoproteins contained in breast milk, co-infection of other enteropathogens, difference in the gut microbiome and histo-blood group antigens(305–309).

2.4.4.2 Indirect vaccine effects

RV vaccination-induced indirect effects have been reported from several countries soon after the implementation of the vaccines. In the US, studies utilizing several different registries have shown supporting data on the reduction of the disease burden also in the unvaccinated population of children and adults. Baker et al.(303) used a time series analysis on insurance diagnosis data to estimate the direct and indirect effects of RV vaccination in the US; they found that the indirect VE against RV hospitalization in adults and children above 10 years of age ranged from 35 to 56 %. Lopman et al.(310) estimated that vaccinations averted 10,000 RVGE hospitalizations in the unvaccinated 5-24-year-old age group in 2008. Similarly in Belgium, the number of RV cases in children aged 10 years and older reduced by a half after vaccine introduction(300). In Austria, mass vaccination program decreased nosocomial and community-acquired RV infections in neonates and unvaccinated children under 42 days old(311). However, in low-income settings, studies have not shown the indirect benefits of RV vaccination on a similar scale. In Malawi, Bennett et al.(312) reported a reduced risk against RV infection for a year after vaccine introduction, but the effect was lost when the study period was extended or in cases

of severe disease. In Rwanda, the number of hospitalizations decreased by a third in the older children after implementation of the RV vaccine(313). By contrast, in studies conducted in Zambia and South Africa, no indirect protection was detected(314,315).

2.4.4.3 Effects on circulating strains

Assessing vaccine effects on circulating RV strains is not straightforward, as it is difficult to distinguish them from natural strain fluctuation, which is multifactorial and mainly unknown. Both individual- and population-level homotypic and heterotypic immunity against RV is perhaps the most important factor causing changes in the circulating genotypes by increasing immune pressure, which may eventually lead to replacement of the predominant strain(151,287). Other factors affecting strain circulation are increased mobility and the migration of people, genetic mutations and host factors as previously discussed.

Several studies from different regions have reported changes in the circulating genotypes after the implementation of a RV vaccine. Soon after the introduction of the Rotarix vaccine in Brazil in 2006, G2P[4] became the predominant strain(316,317). It was speculated that the predominance was due to the previously reported lower efficacy of Rotarix against partly heterotypic G2P[4]; however, a broader examination showed that the prevalence of the strain was already increasing in Brazil and the surrounding countries during and before implementation, supporting natural fluctuation(161,318–320). Predominance of G2P[4] in Brazil continued for a total of five years and was then replaced by alternating G3 and G12 strains(321). Phylogenetic studies did not find signs of selective pressure, but instead the G2P[4] strains found at the beginning and end of the predominance were genetically distinct, supporting the natural fluctuation theory(322,323).

G2P[4] has also become predominant in other countries using Rotarix as well. In Belgium, the strain became predominant a few years after the introduction of the vaccine, and it has remained predominant since, even though the prevalence has decreased in neighboring countries without high-coverage RV vaccination programs(189,324). The strain has similarly become predominant after the introduction of the vaccine also in the Australian territories using Rotarix, but it shares dominance with a novel, similarly partially heterotypic equine-like G3P[8] strain(325,326). Matthijnssens et al.(327) reported that in Belgium, G2P[4] was more prevalent in vaccinated children hospitalized due to RVGE, indicating a possible selective pressure from the vaccine. Using Belgian pre- and post-vaccination data

and mathematical modelling, Pitzer et al.(328) concluded that the Rotarix vaccine had indeed partially influenced to the prolonged predominance of G2P[4], as the data suggested that both natural and vaccine-induced immunity were weaker against heterotypic strains compared to homotypic strain.

In the Australian territories using RotaTeq, G12P[8] has become dominant (326). A similar change has been reported in the US, where G1P[8] was first substituted by G3P[8] and then by G12P[8], which has predominated ever since(329). In fact, a similar transition was detected in Australia(325). A recent study by Ogden et al.(330) described several differences in the antigenic epitopes between G12P[8] strains detected in the US and RotaTeq vaccine strains which may explain the increased prevalence in vaccinated children(330).

Another interesting vaccine-related effect is the biennial incidence pattern seen in the US soon after the introduction of the RotaTeq vaccine(298,303). A similar phenomenon has not been reported in other countries with a high RV vaccine coverage, such as Belgium and Finland(8,300). However, in a recent study, Shah et al.(331) reported that as the number of RV cases in the US has decreased, the magnitude of biennial seasonality has also decreased. The authors speculated that the increase in the previously fairly low (up to 73 %) vaccine coverage may have been the cause and could eventually lead to the elimination of the biennial pattern(331,332).

2.4.4.4 Potential genetic pressure due to rotavirus vaccines

At the beginning of the large-scale introduction of RV vaccines, it was speculated that vaccines would cause increased genetic pressure on the circulating strains, eventually leading to escape mutants and lower vaccine efficacy(333). The outward-facing amino acid sites of VP7 and VP4 proteins have been shown to be vulnerable to genetic pressure which has led to mutations affecting antibody binding and increasing potential for escape neutralization(334). In addition, there are several reports of genetic variance detected in the genotypes during the post-vaccination period. In Belgium, an analysis of VP7 and VP4 proteins of the epidemiological samples by Zeller et al.(335) revealed that, already at the beginning of mass vaccination, the genotype lineages contained by the two vaccines differed from the circulating strains, and a substantial amount of these changes are located at the antigenic sites. In a more recent and more comprehensive whole genome analysis of the G1P[8] strains from the pre- and post-vaccine period, Zeller et al.(336) reported that lineages similar to the Rotarix G1P[8] strain were reduced, possibly due to

vaccine implementation, but no other evidence of selection pressure was apparent. Similar to the case in Belgium, da Silva et al.(337) have reported no sign of genetic pressure in G1P[8] over a 27-year follow-up period in Brazil.

In the Australian territory of Victore, which uses RotaTeq, a post-introductional increase of G1 (lineage 2) RVs was detected when compared to samples collected before the vaccine's introduction(338). An analysis of G1P[8] VP7 and VP8* covering the 20-year period did not find any changes in the VP7 sequences, but by contrast VP8* had several substitutions also in the epitope regions. Also, the P[8] lineage changed from 1 to 3 after the introduction of RotaTeq. However, these changes did not express genetic pressure by the vaccine; they were more or less part of natural fluctuation and reassortance.(339) Hemming et al.(339) speculated that these minor changes seen in G1P[8] could be related to a less pronounced neutralizing antibody response caused by RotaTeq in comparison to the human G1P[8] strain of Rotarix. An exchange of a whole gene was reported by Bucardo et al.(340) in Nicaragua, where two vaccinated children were reported having a breakthrough infection caused by human wild-type G1P[8] RV, of which the NSP2 gene originated from the RotaTeq vaccine strain.

2.4.5 Vaccine safety

Intussusception

IS refers to telescope-like overlapping of one part of the intestine into another, causing obstruction, venous congestion and edema, resulting in ischemia that without treatment may lead to intestinal necrosis, and even perforation and peritonitis(Fig. 3). Natural incidence of IS begins to increase after the first month of life and is at highest at the age of 4-6 months, which coincides with the timing of RV vaccines(341).

As previously discussed, the first licensed RV vaccine, RRV-TV, was withdrawn after nine months of use due to an increased risk for IS. In a case-control study by Murphy et al.(6), vaccine recipients had an approximately 22-fold higher risk for developing IS 3-14 days after the first dose of the vaccine in comparison to unvaccinated infants. The increase in risk for IS was also three times higher 3-14 days after receiving the second dose of the RRV-TV vaccine. It was estimated that continuation of the vaccination would have caused one excess case of IS per 5,000-10,000 vaccinated children.(6) The cause of IS due to the RRV-TV vaccine has remained unknown, but the age of the recipients has been shown to be an important

factor, as 80 % of the IS cases reported by Murphy et al.(6) were seen in 50 % recipients aged at least three months at the time of the first dose, overlapping with the natural increase of the incidence of IS(342–344).

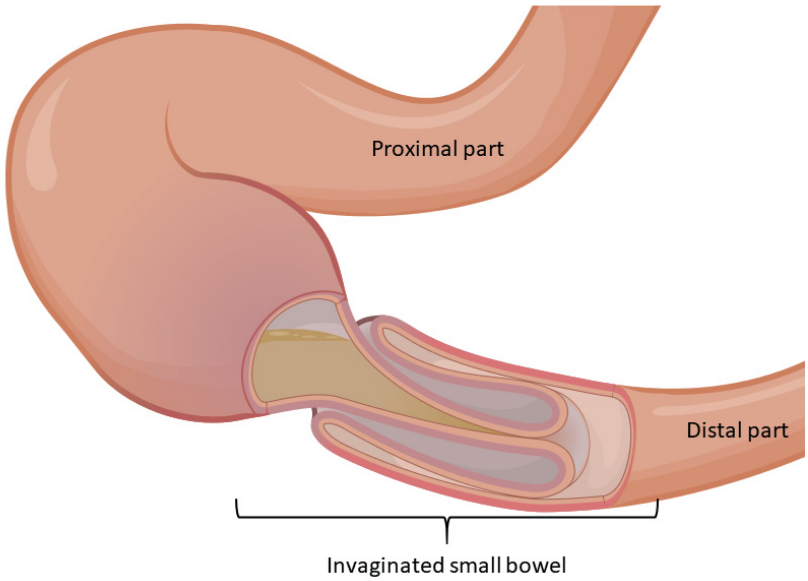

Figure 3. Schematic of intussusception where sections of small bowel have overlapped causing obstruction and venous congestion. Schematic by Olek Remesz, adapted under the Creative Commons Attribution 3.0 Unported License(40).

Pre-licensure studies of the RotaTeq and Rotarix RV vaccines contained IS as the primary safety objective, showing no or a minimal increase in the risk for IS between vaccine and placebo recipients(160,161). However, several post-licensure studies have detected a small increase in the risk for IS after receiving either of the RV vaccines. In Finland, the reported increase was one excess case per 96,000 children for the first 21 days after the first dose of the RotaTeq vaccine but no further increase after the later doses(345). Similar small increases of the incidence of IS have also been reported in Australia and the US with both of the vaccines, and in England with Rotarix(346–350). Even though the current studies have shown that the present RV vaccines are in fact associated with a small increase in the risk for IS, the global consensus is that the benefits – the averted deaths and hospitalizations – outweigh the risk of IS greatly(351,352).

In 2010, both of the current RV vaccines, Rotarix and RotaTeq, were found to be contaminated with the DNA of adventitious viruses. Rotarix was found with porcine circovirus 1 (PCV1) DNA, possibly carried over in porcine pancreas-derived trypsin that was used in passaging Vero cell cultures. RotaTeq was found with a small quantity of simian retrovirus 1 (SRV1) originating from Vero cells.(353) Other studies also reported a small number of fragments of PCV 1 and PCV 2 DNA in the RotaTeq vaccine(354,355), and the origin was confirmed to be the trypsin used(356). The high number of infectious PCV1 in the vaccine led to the development of PCV-free Rotarix, which is currently ongoing clinical studies. Hence, neither the Rotarix nor RotaTeq vaccine, currently in use, are explicitly PCV-free.

2.4.6 RotaTeq vaccine-derived double-reassortant strain

Recombination of the viruses contained in the RotaTeq vaccine was reported for the first time already in a safety and efficacy study of the quadrivalent form of the forthcoming vaccine. During the study, four children were detected shedding recombinant RV, which consisted of human surface proteins G1 and P[8] on a bovine WC3 backbone.(275) Re-reassortance was not further studied in pre-licensure studies, probably as it was kept as a sign of successful vaccine replication in the gut, while two of the vaccine strains (G1P[5] and G6P[8]) infected the same enterocyte and the outer VP7 genes were exchanged.

Since then, the increased virulence of the vdG1P[8] strain has been reported. In 2009, Payne et al.(357) reported symptomatic transmission of a vdG1P[8] from a vaccinated younger sibling to an unvaccinated older sibling who visited an emergency department and received intravenous rehydration due to RVGE. A stool sample of the child was ELISA positive for RV, and further RT-PCR and genotyping showed RotaTeq G1 VP7 and RotaTeq P[8] VP4 on a WC3 bovine RV backbone, similar to that reported by Clark et al.(275) five years earlier. The younger sibling, who had received the RotaTeq vaccine 10 days before the onset of symptoms, was considered the only possible route for transmission. Hemming et al.(358) noted three cases of infants who developed AGE symptoms up to a week after receiving the first (two cases) or the second (one case) dose of the RotaTeq vaccine. After RT-PCR and sequencing vdG1P[8] was detected in the stools of all the children. In another case report by Hemming et al.(359), vdG1P[8] was detected in the stools of a seven-year-old child who was hospitalized for intravenous dehydration due to RVGE. In

this case, the transmission pattern remained unknown as the older siblings were unvaccinated and the younger vaccinated sibling was already two-years old. Six symptomatic vdG1P[8] cases were also detected in Australia among more than 500 samples collected as part of an Australian three-year RV surveillance (2007-2010). Of these samples, four were collected from children showing gastroenteritis symptoms after receiving the RotaTeq vaccine and two were collected in an emergency department setting due to severe AGE(360).

2.4.7 Shedding of rotavirus vaccine strains

As both of the vaccines contain live RVs replicating in the enterocytes of the small intestine, the progeny viruses are shed in stools. Detection of vaccine strains has been used as one marker for vaccine uptake since early RV vaccine studies. Both vaccine strains are occasionally found in stool samples collected from both symptomatic and asymptomatic individuals in RV or AGE surveillance(8,329,361,362). Prolonged shedding obviously indicates prolonged intestinal infection, but the significance of such a chronic infection is unknown.

2.4.7.1 RotaTeq

RotaTeq strain shedding was studied in the pre-licensure studies and as reported low, as only 32 out of 360 (9 %) recipients were shedding four to six days after the first dose of the vaccine. The numbers were even lower after the following doses, as no shedding was detected after the second dose, and only one out of 385 (0.3 %) children after the third vaccine dose was detected with the vaccine virus in the stool sample.(363) Shedding was also assessed in several studies conducted on RotaTeq precursors, with none of them showing higher rates compared to the final product(364). In the developmental and pre-licensure studies on RotaTeq, shedding was studied using cell culture plaque assay, an insensitive method in comparison to RT-PCR. In a later partial re-analysis by Matson et al.(365) using RT-PCR for bovine VP6, shedding was detected in half (65 out of 130) of the recipients after the first dose.

Significantly higher rates of shedding have been identified in more recent post-licensure studies, specifically designed to study the excretion of the RotaTeq vaccine viruses in the stools after inoculation. In these studies the rate of shedding after the first vaccine dose has varied from 21 % (ELISA) up to 94 % (real-time RT-PCR)

depending on the method of detection(366–368). Genotypes related to shedding have been studied a little. In the previously mentioned safety and efficacy study for the quadrivalent form of the vaccine by Clark et al.(275), 7 out of 161 (4.3 %) children were detected shedding, and of those, five were detected with G6P[8] and two with vdG1P[8] in the stools. A recent but more methodologically focused study by Higashimoto et al.(369) found G1 the most commonly shed genotype, followed by G6. However, the study population consisted of only 12 children and it did not include P-typing.

In pre-clinical studies, the presence of the RotaTeq vaccine viruses in stools was studied typically once during three to seven days after inoculation without further surveillance on the total duration. However, the vaccine strains replicate in a similar manner but possibly not at a similar rate as wild-type RVs, and therefore the presence may be even longer than reported. In a large RV surveillance study conducted in Australia by Ye et al.(368), prolonged (>14 days) shedding was reported up to 14 weeks after receiving the third dose of the RotaTeq vaccine with the median duration of 3, 1.5 and 1 week after the first, second and third dose of the vaccine, respectively. In another systematic study by Hsieh et al.(367), the prolonged presence of vaccine viruses was detected only after the first dose, continuing up to 28 days post-inoculation, and in only 11 % of the recipients.

In immunocompromised children, vaccine-derived RV strains may be present even longer and cause difficult symptoms. Patel et al.(370) reported three cases of vaccinated children with severe combined immunodeficiency. Of these children, all had received at least one dose of the RotaTeq vaccine, after which they developed persistent diarrhea leading to failure to thrive and eventually to a diagnosis of severe combined immune deficiency. At its longest, the RotaTeq vaccine strain was present at the age of 13 months, 11 months after the administration of the latest vaccine, dose and it was cleared only after stem-cell transplantation.(370) Other supporting reports have been published since(371,372). Severe combined immunodeficiency-related prolonged replication in the gut has also been shown to lead to mutations in the antigenic epitopes of VP7 and VP4, but the effects of these changes have not been established(373).

2.4.7.2 Rotarix

Shedding of Rotarix was not assessed in the main licensure study(161). However, in pre-licensure studies, the human RV strain, RIX4414, showed a greater tendency to replicate and be shed into stools in comparison to bovine-human reassortant strains

of RotaTeq(364). The shedding rates detected by ELISA seven days after the latest dose varied depending on the given inoculum, ranging from 21 to 80 % after the first dose of the vaccine and from 11 to 24 % after the second(265,267,269,374,375). In a more recent study using real-time RT-PCR for detection, Hsieh et al.(367) have reported a shedding rate of 94 % after the first dose of Rotarix and 53 % after the second.

Prolonged shedding of the Rotarix strain was reported in the pre-licensure studies. In a large Latin American study, a single recipient was detected with the vaccine strain in stools at the time of the second inoculum, 60 days after the first dose(375). However, Phua et al.(267) noted that approximately one child out of eight continued to shed at least 15 days after receiving the second dose of RIX4414, but the exact duration was not determined. In a post-licensure study with 28 days of sampling after both doses, 20 % and 8 % of the recipients, respectively, were shedding for longer than 14 days. In a retrospective RV surveillance study conducted in Scotland, the longest period from inoculation to detection was 43 days(376). As with RotaTeq, prolonged diarrhea has been reported in immunocompromised (SCID) Rotarix recipients(372,377).

Transmission of the Rotarix virus from a vaccinated to an unvaccinated child was reported in a study by Phua et al.(267), where three placebo recipients were detected with the vaccine strain in stools, of which two out of three also seroconverted. In another study by Dennehy et al.(374), two placebo recipients with vaccine group siblings were also detected with the vaccine strain and were seroconverted. In a randomized placebo-controlled study, asymptomatic transmission of the vaccine virus was detected in fifth of sibling pairs(378). The authors did not find genetic changes in comparison to vaccine recipients whereas amino acid mutations have been reported in a case of symptomatic sibling transmission possibly increasing virulence(378,379).

3 AIMS OF THE STUDY

The aims of the present study were the following:

1. To study the prevalence and duration of the shedding of human-bovine reassortant rotavirus vaccine (RotaTeq) strains in vaccine recipients and to assess symptoms related to shedding

2. To study rotavirus genotypes associated with vaccine virus shedding and, specifically, the role of the vaccine-derived double-reassortant G1P[8] rotavirus

3. To examine the genetics of shed G1 vaccine viruses during prolonged multiplication

4. To determine current rotavirus disease burden in all age groups in Finland after four to nine years of mass-vaccination

5. To investigate changes in circulating wild-type rotavirus genotypes after universal rotavirus vaccination in Finland

4 MATERIAL AND METHODS

4.1 Material collection

4.1.1 Study I

The study material was part of a larger prospective study(203,380), investigating the etiology of AGE and respiratory tract infection (RTI) in children, conducted at Tampere University Hospital from September 1, 2009 to August 31, 2011. The study protocol was approved by the Ethics Committee of Pirkanmaa Hospital District. All children under 16 years of age seen in the emergency room or admitted to a pediatric ward with AGE symptoms and admitted children with RTI symptoms were eligible for the original study. Written informed consent was obtained from a parent or legal guardian before enrollment to the study.

For Study I, children with only RTI symptoms were included in the analysis. In total 944 children with RTI symptoms were recruited and 557 (59.0 %) stool samples were obtained. Of those 557 children, 182 (32.7 %) had received at least one dose of the RotaTeq vaccine and formed the study material.

A stool sample was collected from all children during the hospital stay or, if not successful, at home within two weeks with a provided sampling kit. The sample was shipped to laboratory and stored at -20°C until further analysis. The RV vaccination status (vaccine received, number of doses and dates of dosing) of each child was inquired from the respective child welfare clinics by a study nurse.

4.1.2 Study II

A prospective study was conducted at three vaccine research clinic sites in Finland (Espoo, Tampere and Turku) from August 2015 to February 2019. The study protocol (EudraCT 2014-004252-60) was approved by the Ethics Committee of the Hospital District of Southwest Finland and by the Finnish Medicines Agency. All children who received the RotaTeq vaccine according to the Finnish NIP at their

local child welfare clinic were eligible for the study. Informed consent was obtained from the parents by study nurse.

A total of 301 children were enrolled (Fig. 4). At least two stool samples were collected from the participants; 292 samples 5-10 days after the first dose and a second sample was collected from 247 children 0-7 days before receiving the third dose of the RotaTeq vaccine. In 50 children, the second stool sample was positive for RV VP7 and a further third sample was obtained from 42 of those children six weeks after receiving the third vaccine dose. Similarly, a fourth stool sample was received from 9 out of 11 children who were detected with RV VP7 in the third stool sample. The fourth sample was taken a total 12 weeks after the third vaccine dose. Parents were provided with a stool sample kit for sampling at home and for the delivery of the sample by mail. A voluntary serum sample was requested from the parents of children who were detected to be RV positive in the third or fourth stool sample.

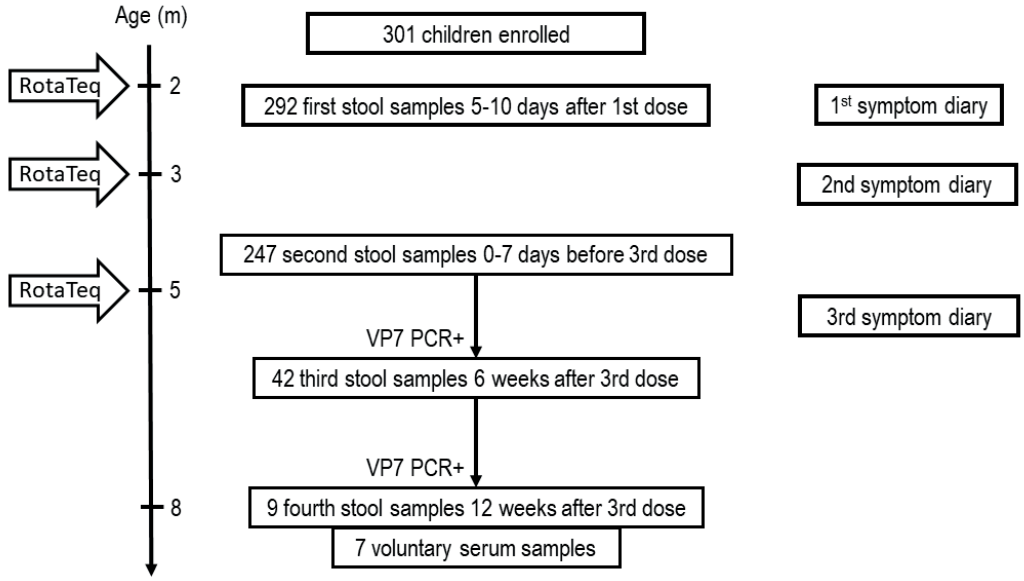

Figure 4. Flowchart of Study II.

A diary card on possible symptoms following vaccination was filled by parents after each dose of the RotaTeq vaccine for seven days (vaccination at day one). The diary inquired temperature of the child (either axillary or rectal), maximal number (0, 1, 2-4 and ≥5 times) of vomiting episodes per day and maximal number (0, 1-3, 4-5 and ≥6 times) of diarrheal stools per day. Parents could also record other observations.

The severity of diarrheal symptoms was scored according to the 20-point Vesikari score system(106). Total of 284, 194 and 227 symptom diaries were received after the respective vaccine doses, from which 278, 182 and 224, respectively, were properly filled and eligible for further analysis.

4.1.3 Studies III-IV

Studies III and IV used the same study material collected in collaboration with the National Institute for Health and Welfare (THL). In 2013, RV was included as part of the microbe strain collection under the Finnish Communicable Diseases Act and Decree. Since then, all clinical laboratories in the country have been obliged to forward all RV positive samples to THL, from which the samples have been further referred to the Vaccine Research Center at Tampere University for genotyping. RV detection in clinical laboratories has mainly been based on ELISA but also to some extent on multiplex real-time PCR.

Study III is based on two RV seasons from September 2013 to August 2015, during which 405 stool samples were received for genotype analysis. Study IV includes expanded material with 755 samples collected during the following three RV seasons up to August 2018, and the total number of samples in Study IV was 1160. The samples were divided in half in THL, shipped to the Vaccine Research Center and stored at -20°C until further analysis.

The following information from the RV cases was available: age with the accuracy of the year, gender, sampling date, original laboratory result from the clinical laboratory and location of the clinical laboratory, from which the sample was forwarded to THL. In some cases, additional information was included when the sample was referred for typing, such as RV vaccination status, travel history, or description of symptoms.

4.2 Laboratory methods

4.2.1 Sample preparation

Stool samples (I-IV)

A 10% stool suspension was prepared in phosphate-buffered saline (PBS). The mixture was then vortexed, incubated at room temperature for 15 min and centrifuged at 14,700 x g for 5 min. The supernatant was collected for further use and stored at -20°C. In the case of diaper samples, the most representative part of the diaper was used instead of the stool and processed in a similar manner.

Serum samples (II)

Some 2-3ml of whole blood was taken by venous puncture into a separation tube. The tube was inverted at least six times and incubated at room temperature for at least 1 hour. The samples were then centrifuged at 1,000-1,300 x g for 10 min. After which, the separated serum was transferred into sterile tubes and stored at -20°C.

4.2.2 RNA extraction (I-IV)

Viral RNA was extracted from the 10 % stool suspensions using a QIAGEN QIAamp Viral RNA Mini Kit (Hilden, Germany) according to manufacturer's instructions. Briefly, 140 µl of 10 % stool suspension was mixed with 560 µl of Buffer AVL-Carrier RNA solution, vortexed shortly and incubated at room temperature for 10 min. Then 560 µl of 99.5 % ethanol was added to the mixture, of which, after a short vortex, 630 µl of the mixture at a time was transferred into a spin column and centrifuged at 6,000 x g for 1 min. The supernatant was discarded, and the column was washed by centrifuging 500 µl Buffer AW1 and AW2 at 6,000 x g for 1 min and at full speed for 3 min, in the respective order. Extracted viral RNA was then eluated to Buffer AVE after a 1 min incubation at RT and 1 min of centrifugation at 6,000 x g. Viral RNA extracts were stored at -70°C.

4.2.3 RT-PCR (I-IV)

4.2.3.1 VP7 detection and G-typing by RT-PCR

First, 5µl of extracted viral RNA was combined with 2 µl of a primer mixture of Rota Beg9 fwd and Rota End9 rev (Table 4). The mixture was then denatured in thermal cycler at 94°C for 2 min and kept on a cold block. The RT-PCR reaction mix was prepared by combining 1.8 µl of nuclease free water, 1.2 µl of 25 mM $MgCl_2$ (Promega, Wisconsin, USA), 1 µl of 2.5 mM dNTP mix (Promega), 3 µl of 5 x Green Go Taq Flexi Buffer (Promega), 0.5 µl of AMV RT-enzyme (Promega) and 0.5 µl of RNasin ribonuclease inhibitor (Promega) per reaction. Then 8µl of RT-PCR mix was added to the denatured sample-primer mix. Primers were attached by incubation at 42°C for 60 min. Samples were kept at 8°C, and a 1st PCR mix was prepared. The mixture consisted of 20.6 µl of sterile aqua, 10 µl of 5 x Green GoTaq Flexi Buffer, 2 µl of 25 mM $MgCl_2$, 2µl of 2.5 mM dNTP mix and 0.4µl of GoTaq DNA polymerase (Promega). Then 35 µl of 1st PCR mix was mixed with the samples and a PCR program was run as follows: 94°C for 3 min, 35 cycles of 94°C for 20 sec, 56°C for 1 min and 72°C for 2 min, followed by a 5 min step at 72°C and a final hold at 8°C.

For G-typing the samples, a 2nd PCR reaction with two different primer mixes (H and C pool mix) was conducted (Table 2). The 2nd PCR mix consisted of 24.8 µl of sterile aqua, 10 µl of 5 x Green GoTaq Flexi Buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM dNTP mix, 6 µl of H or C pool primer mix and 0.2 µl of GoTaq DNA polymerase with a total volume of 48 µl. Then 2 µl of 1st PCR products were added to 2nd PCR mix and the following PCR program was run: 94°C for 3 min, 25 cycles of 94°C for 15 sec, 53°C for 40 sec and 72°C for 70 sec, followed by a 5 min step at 72°C and a final hold at 8°C. Then 10 µl of the final PCR products (1st, 2nd C and H pool) was pipetted into 2 % agarose gel with a molecular marker (GeneRuler 100 bp DNA Ladder, Thermo Fischer Scientific, Massachusetts, USA) and run for 105 min at 100 V. The gel was then UV-illuminated, and the amplicons defined by length (Table 4).

Table 4. Oligonucleotide primer sequences and amplicon sizes for RV G-typing RT-PCR.

Primer name	Sequence (5'-3')	Amplicon size (bp)
Rota Beg 9 fwd	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1062
Rota End 9 rev	GGTCACATCATACAATTCTAATCTAAG	
H pool mix		
Rota H rev	AACTTGCCACCATTTTTTCC	
Rota hG1 fwd	CAAGTACTCAAATCAATGATGG	618
Rota hG2 fwd	CAATGATATTAACACATTTTCTGTG	521
Rota hG3mod fwd	ACGAACTCAACACGAGAGG	682
Rota hG4 fwd	CGTTTCTGGTGAGGAGTTG	452
Rota hG8 fwd	GTCACACCATTTGTAAATTCG	754
Rota hG9mod fwd	CTTGATGTGACTAYAAATAC	179
Rota hG12 fwd	CCGATGGACGTAACGTTGTA	387
C pool mix		
Rota C fwd	TAGCTCCTTTTAATGTATGG	
Rota cG1mod rev	CCATCATTGATTTGAGTACTTG	298
Rota cG2 rev	GTTAGAAATGATTCTCCACT	244
Rota cG3mod rev	CTGTTGCAACTTCTTCAAACG	672
Rota cG4 rev	GGGTCGATGGAAAATTCT	403
Rota cG8 rev	CGAATTTACAAATGGTGTGAC	161
Rota cG9mod rev	TATAAAGTCCATCGCACTAG	110
Rota cG12 rev	TACAACGTTACGTCCATCGG	529

bp = base pair

4.2.3.2 VP4 detection by RT-PCR

A RT-PCR mix was prepared by combining 1.6 µl of Nuclease free water, 1.5 µl of 10 x PCR buffer II (Applied Biosystems, Foster City, CA), 1.2 µl of 25mM $MgCl_2$ (Applied Biosystems), 1.2 µl of 2.5 mM dNTP mix (Promega), 2 µl of AMV RT-enzyme (Promega) and 0.5 µl of RNAsin (Promega). A primer mixture of Rota VP4 fwd and rev (1 µl of each) was combined with 5 µl of sample RNA and the mixture was then incubated at 94°C for 2 min. (Table 5)

Then, a 1st PCR mixture was prepared consisting of 24.25 µl of sterile aqua, 3.5 µl of 5 x Green GoTaq Flexi Buffer, 4.2 µl of 25 mM $MgCl_2$, 2.8 µl of 2.5 mM dNTP mix and 0.25 µl of GoTaq DNA polymerase. The prepared volume of 35 µl of the mixture was added to the incubated samples and the following PCR program was run: 94°C for 3 min, 30 cycles of 94°C for 20 sec, 50°C for 1 min and 72°C for 1 min, followed by a 5 min step at 72°C and a final hold at 8°C.

P-typing was conducted by a 2nd PCR reaction. Some 2 µl of the 1st PCR product was combined with 2 µl of P pool primer mix (Table 5), 28.6 µl of sterile aqua, 10 µl of 5 x Green GoTaq Flexi Buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM dNTP mix and 0.4 µl of GoTaq DNA polymerase. The primer-sample mixture was then moved to a thermomixer and the following PCR program was run: 95°C for 2 min, 25 cycles of 94°C for 35 sec, 45°C for 30 sec and 72°C for 70 sec, followed by a 5 min step at 72°C and a final hold at 8°C. Then 10 µl of the final PCR product (1st and P pool) was pipetted into 1.5 % agarose gel with a molecular marker (GeneRuler 100 bp DNA Ladder) and run for 90 min at 100 V. The gel was then UV-illuminated, and the amplicons defined by length (Table 5).

Table 5. Oligonucleotide primer sequences and amplicon sizes for RV P-typing RT-PCR.

Primer name	Sequence (5'-3')	Amplicon size (bp)
Rota VP4 fwd	TATGCTCCAGTNAATTGG	664
Rota VP4 rev	ATTGCATTTCTTTCCATAATG	
P pool mix		
Rota VP4 fwd2a	GATGGTCCDTATCARCC	
Rota VP4 P[4] rev	CTATTGTTAGAGGTTAGAGTC	289
Rota VP4 P[6] rev2	AATTTGAAGTTGACGAGTA	381
Rota VP4 P[8] rev	TCTACTGGRTTRACNTGC	151

bp = base pair

4.2.3.3 VP6 detection by RT-PCR

Bovine rotavirus VP6 (I)

Some 5 µl of sample RNA was mixed with 5 µl of Molecular Biology Grade Water (Sigma Aldrich, Missouri, USA) and the sample was denatured at 95°C for 2 min. A total volume of 40 µl of RT-PCR mix per reaction was prepared consisting of 18 µl of Molecular Biology Grade Water, 10 µl of 5 x OneStep RT-PCR buffer, 2 µl of dNTP mix (10 mM each), 4 µl of Rota VP6 fwd Bovine primer, 4 µl of Rota VP6 rev Bovine primer and 2 µl of OneStep RT-PCR enzyme mix. (Table 6) The denatured sample RNA and RT-PCR mix was combined on ice and the following PCR program was run: 50°C for 30 min, 95°C for 15 min, 40 cycles of 94°C for 45 sec, 54°C for 45 sec and 72°C for 1 min, followed by a 10 min step at 72°C and a final hold at 8°C.

Then 10 µl of the final PCR products were then pipetted into 1.5 % agarose gel with a molecular marker (GeneRuler 100 bp DNA Ladder) and run for 90 min at 100 V. The gel was then UV-illuminated, and the amplicons were defined by length (Table 6).

Whole-genome VP6 (II-IV)

The protocol was similar to the previously described bovine RT-PCR protocol with the following differences. The RT-PCR mix consisted of 20 µl of Molecular Biology Grade Water, 10 µl of 5 x OneStep RT-PCR buffer, 2 µl of dNTP mix (10 mM each), 6 µl of Rota FG P6 fwd primer, 6 µl of Rota FG VP6 rev primer and 2 µl of OneStep RT-PCR enzyme mix. (Table 6) The PCR program also had some minor differences: 45°C for 30 min, 95°C for 15 min, 40 cycles of 94°C for 45 sec, 45°C for 45 sec and 68°C for 1 min, followed by a 10 min step at 72°C and a final hold at 8°C.

Table 6. Oligonucleotide primers and amplicon sizes for RV VP6 RT-PCR.

Primer name	Sequence (5'-3')	Amplicon size (bp)
Rota VP6 fwd Bovine	GAYGGNGCDACNACATGGT	379
Rota VP6 rev Bovine	GTCCARTTCATNCCTGGYGG	
Rota FG VP6 fwd	GGCTTTWAAACGAAGTCTTC	1356
Rota FG VP6 rev	GGTCACATCCTCTCACT	

bp = base pair

4.2.4 Sequencing and sequence analysis (I-IV)

DNA size-selection by electrophoresis

A 2 % agarose gel with 1 x TAE buffer was prepared and PCR products were loaded into wells. The gel was run for 90 min at 100 V. After electrophoresis, DNA bands were size-selected under UV illumination, cut and weighted.

DNA was then extracted from the agarose gel using a QIAgen QIAquick Gel Extraction Kit (QIAgen, Hilden, Germany) as follows. Three volumes of Buffer QG were added to one volume of gel, and the mixture was then incubated for 10 min at in a 50°C water bath. When the size of the DNA fragment was under 500 bp or above 4kbp, after the gel had dissolved, one gel volume of isopropanol was added.

Then the sample was transferred to a QIAquick spin column and centrifuged at 15,200 x g for 1 min. The flow-through was discarded, 0.5 ml of Buffer QG was added to the column and the column was centrifuged as previously. After discarding the flow-through for the second time, 745 µl of Buffer PE was added and the column was let to stand for 5 min before being centrifuged as previously. The flow-through was discarded and the column was centrifuged for 1 min at 16,100 x g. The column was then moved into a clean 1.5 ml Eppendorf tube and the DNA was eluted to 30 µl of ddH_2O.

Sequencing PCR

Another 2 % agarose gel with 1 x TAE buffer was prepared and loaded with a mixture of 2 µl of purified DNA, 10 µl of ddH_2O and 2.5 µl of loading buffer. The gel was then run for 60-90 min at 100 V, and visualized under UV light. The volume (1-5.5 µl) of DNA of each sample was then determined on the basis of the visual intensity of each band, where 1 µl was used with a strong band and 5.5 µl with a barely visible band. A PCR mixture was prepared on ice, containing: 1-5.5 µl of DNA, 2 µl of BigDye TTR-mix, 3.2 pmol of primer, 1 µl of BDT 5 x Sequencing Buffer and ddH_2O up to a total volume of 10 µl. The reaction was performed using 96-well plate or 8 sample strips. Next, the following PCR program was run: 96°C for 1 min, 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, and hold at 8°C. The same primers were used as in the RT-PCR reactions (Table 4-6).

Ethanol precipitation

To remove extra nucleotides and primers, the samples were purified by ethanol precipitation. First, a mixture of 10 µl ddH_2O, 2 µl of sodium acetate and 50 µl of 99,5 % ethanol per sample was prepared, and 60 µl of the mix was added to each sample well. The plate or strip was then covered, vortexed, and incubated at room temperature for 15-30 min. After centrifugation at 2,000 x g at RT for 45 min, ethanol was removed, and 180 µl of 70 % ethanol was added and the samples were centrifuged at 2,000 x g for another 10 min. Ethanol was again removed by centrifuging the plate upside down at 700 x g for 1 min. The pellets were then resuspended into 15-25 µl of HiDi Formamide and incubated at room temperature for 5-15 min. The samples ready for sequencing were stored at +4°C until sequencing.

Sequencing

Sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI PRISM 310 Genetic Analyzer, and, since June 2018, using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit on an ABI 3500XL Genetic Analyzer.

Sequence analysis

Sequences were analyzed with Sequencher 4.10.1 (Gene Codes Corp Inc., An Arbor, MI, USA) and compared with published reference strains from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/, Nucleotide blast). In the unpublished data RotaTeq G1 VP7 (GenBank accession no. GU565057), RotaTeq P[8] VP4 (GU565044) and RotaTeq VP6 (GU565056) sequences were used as references for comparison.

4.2.5 ELISA

Rotavirus detection in stools (I, II)

RV antigen detecting ELISA in stools was conducted using a commercial RV ELISA kit, ProSpect (Oxoid Ltd, UK) according to the manufacturer's instructions. Briefly, a stool suspension was prepared by adding 100 mg of stool to sample diluent. After mixing and incubating for 10 min at room temperature, the sample was centrifuged at 1,000 x g for 10 min at room temperature.

One hundred microliters of sample was pipetted in two parallel wells coated with a rotavirus specific rabbit polyclonal antibody. Two negative and positive controls were included in each sample plate. After sample addition, two drops of conjugate were inserted into each well and the plate was covered with sealing tape, mixed, and incubated for 60 min at room temperature. After incubation, the wells were washed with Wash Buffer and double-distilled H_2O using Denly Wellwash 4 (Denley Instruments Ltd, UK). Two drops of substrate were added to each of the washed wells, and the plate was mixed and incubated in the dark for 10 min at room temperature. The substrate reaction was stopped by adding two drops of stop solution. The optic density of the samples at 490 nm was then read by a microplate reader Victor^2 1420 (Perkin Elmer, Waltham, MA). The cut-off value was

determined by adding 0.100 absorbance units to the absorbance value of the negative control.

Serum VP6 IgA (II)

Serum RV VP6 IgA levels of children with long-term shedding were determined by ELISA to study if the children were seroconverted by the RotaTeq vaccine. A 96-well plate was coated with 1 μg/ml of rabbit anti-RV group A antibody (GenWay Biotech Inc., California, USA), and in a baculovirus-insect cell expression system produced VP6 antigens were added at a concentration of 1 μg/ml. The wells were blocked with 5 % milk and serially diluted serum samples (from 1:100 to 1:3,200) were added two-fold to the wells. VP6 IgA antibody binding was detected with 1:4,000 diluted horseradish peroxidase-conjugated goat anti-human IgA (Thermo Scientific) and 0.4 mg/ml o-phenylenediamine dihydrochloride substrate (FAST-OPD, Sigma-Aldrich). An optical density at 490 nm was measured by Victor² 1420 (Perkin Elmer, Waltham, MA). A cut-off value was determined by adding the mean absorbance of the negative control to three times the standard deviation of the negative controls, and the value had to be ≥ 0.100.

4.2.6 Cell culture in MA104 cells (I-II)

In Study I, a 10% stool suspension containing 1ml of minimum essential medium (MEM) was vortexed and then supplemented at 3,000 x g for 15 min. The solution was then filtered through a 0.22 filter and diluted in MEM containing 10 % fetal calf serum with trypsin (0.5 μg/ml) (Gibco). The virus-MEM solution was then activated for 30 min in a humified 5 % CO_2 incubator. MA104 cells seeded in a 25 cm² tissue culture flask and six-well plate were washed with trypsin-MEM. The cells were infected with 2ml of virus dilution and incubated for 1h in a CO_2 incubator. After 24 hours, the virus dilution was replaced with a virus culture medium containing MEM with penicillin/streptomycin (1:100) and L-glutamine (1:100) as a supplement. The cell lines were monitored daily for a cytopathic effect. Cells that showed a cytopathic effect were stored at -20°C. Cells were scraped from the wells, harvested and centrifuged at 1,100 rpm for 5 min. Part of this passage 0 supernatant was then transferred into a new 25 cm² flask and six-well plate which were treated with MEM and 0.5 μg/ml trypsin. After 48 hours, the passage 1 cells were scraped, harvested and centrifuged at 1,100 rpm for 5 min. This was repeated for a total of three passages, and the final supernatant was stored at -20°C until used in RT-PCR.

The protocol in Study II was otherwise similar but contained only passage 0, with the difference that the cells were observed for a cytopathic effect for seven days before scraping and harvesting.

4.3 Statistical methods (II, III, IV)

Statistical analyses were conducted using SPSS 23.0 (III) and 25.0 (II and IV) (IBM Corp., Armonk, NY). A p-value <0.05 was considered statistically significant.

Depending on the sample size, Fisher's exact test (III) or the chi-square test (II and IV) was used to determine the difference in genotype distributions of vaccinated and unvaccinated children (III and IV), and the relation of symptoms to the duration of shedding and to the genotypes shed (II). Exact values were calculated when the chi-square test assumptions were not met due to a too high proportion of low number contingency cells. The Mann-Whitney U test (II, IV) was used to calculate differences in the age and genotype distributions.

5 RESULTS

5.1 Shedding of RotaTeq vaccine strain rotaviruses

5.1.1 Prevalence of shedding (I,II)

Study I examined RotaTeq vaccine virus shedding at random time points in children aged 2 to 8 months hospitalized for a RTI showing no gastroenteritis symptoms. In Study I, shedding of RotaTeq vaccine strain VP7 was detected by RT-PCR in the stools of 30 (16.5 %) out of 182 children who had received at least one dose of RotaTeq vaccine. Of these children, 28 had received the first dose of the RV vaccine and 14 (50.0 %) were found to shed the RotaTeq vaccine virus at the time of hospitalization. Some 38 children had received two doses, and of those 10 (26.3 %) were excreting vaccine strains in the stools. Most of the 182 children, 116 (63.7 %), had received all three doses of the vaccine, and only 6 (5.2 %) cases were positive for the RotaTeq vaccine strains. (Fig. 5)

In Study II, shedding of RotaTeq vaccine viruses was detected in 272 (93.2 %) out of 292 children 5-10 days after the first dose of the vaccine. A second stool sample 0-7 days before the third dose was received from 247 children, of whom 50 (20.2 %) were detected with vaccine strain in stools. Long-term shedding six weeks after receiving the third vaccine dose was found in 11 (26.2 %) out of 42 children who provided the third stool sample. Nine of the 11 children provided the last sample and two of those children continued shedding up to 12 weeks after the third dose at the age of eight months. (Table 7)

5.1.2 Duration of vaccine virus excretion (I, II)

Among the children hospitalized for RTI in Study I, the duration of shedding was calculated by subtracting the sampling date from the date of the latest vaccine dose given. In total, prolonged (over 14 days) shedding was detected in 16 (53.3 %) out of a total of 30 cases, with long-term shedding lasting over 30 days in nine (30.0 %)

cases. At its longest, RotaTeq vaccine strain was detected in the stools of one immunocompetent child 84 days after receiving the third dose of the vaccine. The proportion of prolonged shedders increased after each vaccine dose received, as 4 out of 14 (28.6 %) children after the first, 7 out of 10 (70.0 %) after the second, and 5 out of 6 (83.3 %) children after the third dose were shedding for longer than 14 days following the inoculation.

Study II was designed to study long-term shedding and the sampling points were timed to be relevant to the main goal. Fifty (20.2 %) of the 272 children were detected to shed RotaTeq vaccine strain 5-10 days after the first dose of the vaccine and then to continue shedding up to 0-7 days before receiving the third dose. After subtracting the sampling time frame, these children were shedding for a total of approximately 2.5 months. The third sampling point was six weeks after the third dose, and out of 42 children, 11 were detected with vaccine strain, meaning a total shedding time of 4.5 months. Extensively long shedding was found in two children who remained positive for RV vaccine virus six months after receiving the first dose, ergo up to the age of eight months. A voluntary serum sample was requested from these 11 children, of whom seven provided a sample. Out of the seven children, six showed to be seroconverted against serum VP6 IgA. All children were detected with RotaTeq G1 in their stools in all samples. (Table 7)

Viability of long-term shed vaccine-derived rotaviruses by cell culture

Five series of subsequent samples were selected for cell culture in MA104 cells. The RT-PCR results for both original stool samples and cell culture supernatants are shown in Table 8. In three cases (1-3) the first sample was ELISA positive. In the other two cases (4 and 5) ELISA was negative but RT-PCRs remained positive up to the fourth stool sample. The samples were cultivated for seven days without a visual cytopathic effect. In three cases (1-3), RT-PCRs for VP7, VP4 and VP6 of the cell cultured first samples were positive. In two of these cases (1 and 3), the vdG1P[8] strain was successfully cultivated. Some changes in the shed G-types was seen as in one case (2) the RT-PCR findings of the first and second samples (G1+G4+P[8] and G1P[8], respectively) differed from the cell culture findings (G1+G6+P[8] and G6P[8], respectively). In another case (5), the cell culture of the third sample showed G1 similar to the stool sample results; however, the previous cell cultures were (illogically) negative.

Table 7. Summary of detected RotaTeq vaccine genotypes at each time point.

Genotype combinations	Time after the 1st dose, n (%)			
	5-10 d (n=272)	**3 m** (n=50)	**4.5 m** (n=11)	**6 m** (n=2)
G1	43 (15.8)	32 (64.0)	10 (90.9)	2 (100)
G1P[5]	11 (4.0)	1 (2.0)		
G1P[8]	61 (22.4)	10 (20.0)		
G1+P[5]+P[8]	11 (4.0)			
G1+G3+P[8]	2 (0.7)			
G1+G3+G4	1 (0.4)			
G1+G3+G4+P[5]+P[8]		1 (2.0)		
G1+G4	12 (4.4)			
G1+G4+P[5]	18 (6.6)			
G1+G4+P[8]	31 (11.4)			
G1+G4+P[5]+P[8]	12 (4.4)			
G1+G4+G6	1 (0.4)			
G1+G4+G6+P[5]	1 (0.4)			
G1+G4+G6+P[8]	7 (2.6)			
G1+G6	1 (0.4)			
G1+G6+P[8]	7 (2.6)	1 (2.0)		
G1+G6P+[5]+P[8]	1 (0.4)			
G2	1 (0.4)	1 (2.0)		
G2P[5]	1 (0.4)			
G2+G4	1 (0.4)			
G2+G4+G6+P[8]	1 (0.4)			
G3	1 (0.4)			
G3P[8]	1 (0.4)			
G3+P[5]+P[8]	2 (0.7)			
G3+G4	1 (0.4)			
G3+G4+P[5]	3 (1.1)			
G3+G4+P[8]	1 (0.4)	1 (2.0)		
G3+G4+P[5]+P[8]	3 (1.1)			
G4	10 (3.7)	1 (2.0)		
G4P[5]	2 (0.7)			
G4P[8]	4 (1.5)			
G4+P[5]+P[8]	2 (0.7)			
G4+G6	1 (0.4)			
G4+G6+P[5]	1 (0.4)			
G4+G6+P[8]	7 (2.6)			
G6P[8]	7 (2.6)			
P[8] only		1 (2.0)		
VP6 only	3 (1.1)	1 (2.0)	1 (9.1)	

Table 8. RotaTeq vaccine G- and P-types detected in stool samples and cell cultures in five series of cases. VP6 positive samples are bolded.

Case	Sample type	Sample			
		1st	2nd	3rd	4th
1	SS	**G1+G4+P[8]**	**G1P[8]**	**-**	-
	CC	**G1P[8]**	neg	**-**	**-**
2	SS	**G1+G4+P[8]**	**G1P[8]**	**-**	**-**
	CC	**G1+G6+P[8]**	**G6P[8]**	**-**	**-**
3	SS	**G1P[8]**	**G1P[8]**	G1	-
	CC	**G1P[8]**	neg	neg	-
4	SS	**G1P[8]**	**G1**	G1	**G1**
	CC	neg	neg	neg	neg
5	SS	**G1**	**G1**	**G1**	**G1**
	CC	neg	neg	G1	neg

SS = stool sample, CC = cell culture

5.1.3 Genotypes and combinations (I, II)

In Study I, G1P[8] was the most common genotype detected in 8 (57.1 %) out of 14 cases after the first dose of the vaccine. Whereas, after the second and third dose, G1 was most frequently detected. (Fig. 5) Of the 30 cases, bovine-origin VP6 was detected in 29 cases, and a human-origin VP6 in one case – in which the child was found with a combination of RotaTeq G1 VP7 and wild-type P[8] VP4 10 days after receiving the second dose of the vaccine.

Stool RV antigen ELISA was conducted in 19 out of the 30 RT-PCR positive cases, while in the remaining cases the sample type (diaper) or amount was insufficient for analysis. Of the 19 samples, only four (16.7 %) were ELISA positive, all in children detected with vdG1P[8].

All detected genotype combinations in Study II are presented in Table 7. After the first dose (N=272), the most common detections were G1P[8], G1 alone and G1+G4+P[8] which were shed in stools in 61 (22.4 %), 43 (15.8 %) and 31 (11.4 %) cases, respectively. In comparison, the original vaccine strains G1P[5] (N=11), G6P[8] (N=7), G4P[5] (N=2) and G2P[5] (N=1) were much rarer findings. The fifth original strain, G3P[5], was detected only in combination with other G- or P-type. After the second dose, G1 alone had become the most common with 32 (64.0 %)

out of 50 detections, followed by G1P[8] (N=10). Other genotypes were single detections. Likewise, G1 alone was also the only genotype detected 6 and 12 weeks after the third dose of the RotaTeq vaccine, with the exception of one case where only VP6 RT-PCR was positive (Table 7).

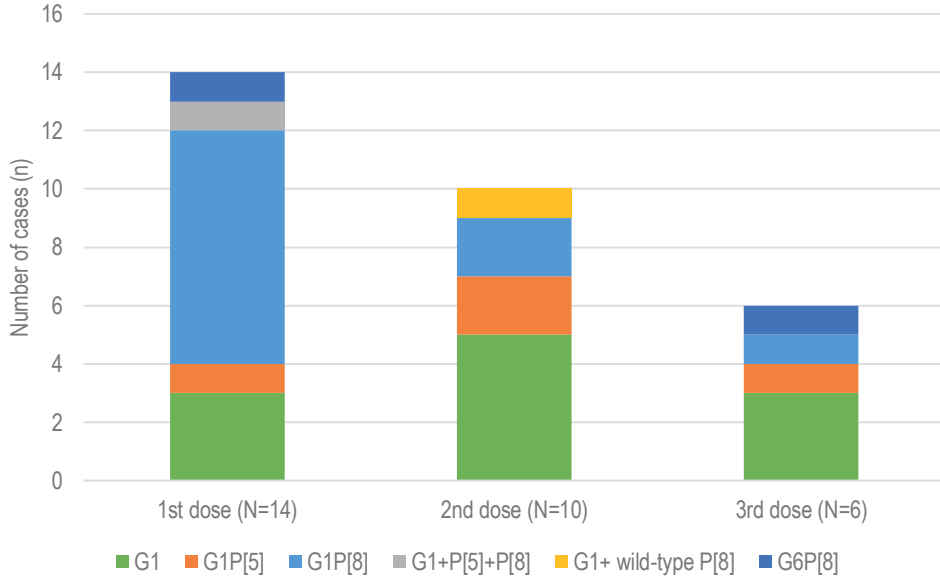

Figure 5. Number and genotypes of RotaTeq vaccine-derived strains detected at random time-points after each dose of the vaccine.

When examining G-types only, G1 was associated in 93.3 % of all the cases in the Study 1 (Fig. 5). In the Study II, G1 was predominant as well, as it was found in 220 (81.8 %) out of 269 VP7 positive first stool samples, in 45 (93.8 %) out of 48 cases just before the third dose, and in each of the later stool samples (Fig. 6). Of the other RotaTeq vaccine contained G-types, G4 was the only common detection found in 120 (44.6 %) of the VP7 positive stool samples after the first dose. Of the P-types, P[8] was by far more common than P[5], being found in 159 (81.1 %) of the 196 VP4 positive cases after the first dose, whereas P[5] was detected in 68 (34.7 %).

Bovine-origin VP6 was detected in 257 (88.0 %) out of the 292 first stool samples provided but only in 37 (15.0 %) of the 247 second stool samples (Fig. 6). As the only detection, VP6 was seen in three cases after the first dose of the vaccine, and in one case just before the third dose and six weeks after the third dose. Only two of the 11 VP7 positive third samples were also VP6 positive; however, both positive fourth samples given 12 weeks after the third dose, were also positive for VP6.

All RT-PCR positive first stool samples were studied by RV antigen ELISA. In 45 cases, the test could not be performed due to the insufficient amount or type of the sample (diaper). Of the remaining 227 samples, 37 (16.3 %) were ELISA positive. The most common finding among the ELISA positive children was vdG1P[8] (N=23, 62.2 %). Other genotypes were G1+G4+P[8] (N=4, 10.8 %), G4+G6+P[8] (N=3, 8.1 %) and G6P[8] (N=2, 5.4 %), whereas G1 alone, G1+G4+P[5], G1+G4+P[5]+P[8], G1+G4+G6 and G1+G4+G6+P[8] were found as single detections. RT-PCR positive second, third, and fourth samples were also tested for ELISA, all being negative.

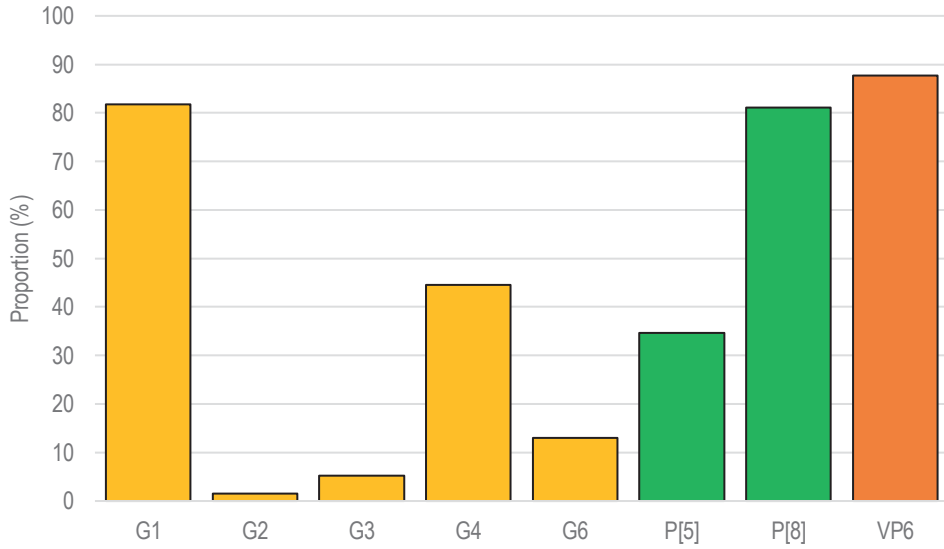

Figure 6. Distribution of different RotaTeq G-types (n=269), P-types (n=196) and VP6 (N=257) in first stool samples (n=292) collected 5-10 days after the first dose of the RotaTeq vaccine.

5.1.4 Shedding of vaccine-derived double-reassortant G1P[8] (I, II, unpublished)

VdG1P[8] was a common finding in both Studies I and II, especially after the first dose of the vaccine. In Study I, vdG1P[8] was detected in eight (57.1 %) children out of 14 after the first dose of the vaccine, and in 11 (36.7) children out of 30 in total. All of these children were hospitalized for RTI and had no AGE symptoms. Likewise, in Study II, vdG1P[8] was the most common detection right after the first

dose with 61 (22.4 %) out of 272 detections, and it was also detected in 10 (20.0 %) of the 49 second samples.

Cell culture of vdG1P[8]

In the unpublished data from Study I material, stool samples of three children detected with RotaTeq vdG1P[8] were cultured in MA104 cells. The children had recently received the first dose of RotaTeq vaccine and were ELISA positive. The samples were obtained six days (one case) and 14 days (two cases) after the vaccination. Two of the samples showed no sign of growth in cell culture, but in one case (sample obtained six days after vaccination), the virus was successfully propagated and remained stable in three passages of cell culture, indicating presence of a viable virus potentially acting as a pathogen. In comparison, in Study II, in two cases the vdG1P[8] strain was grown successfully in cell culture, which are previously described in section 5.1.2 and presented in Table 8.

5.1.5 Genetics of long-term shedding (unpublished)

Analysis of G1 VP7 sequences

At least two subsequent G1P[8] or G1 VP7 sequences were available in 38 cases of the Study II material. In comparison to RotaTeq G1 VP7 sequence, no nucleotide level changes were found in the first stool samples, collected 5-10 days after the first dose of the vaccine. In 27 (71.1 %) of the 38 second stool samples, at least one nucleotide substitution was observed. In the majority (21 out of 27) of the cases, a transition of purines A to G was found. This transition caused an amino acid change of Aspartate to Asparagine at position 145, which is located in the epitope region 7-2. In addition, in one sequence, there were two other amino acid changes, at position 129 (Valine to Alanine) located in epitope region 7-1a, and at amino-acid position 37 (Phenylalanine to Leucine), which is located outside epitope regions. Of other single-point nucleotide mutations, there was a transition of purines G to A causing amino acid substitution from Aspartic acid to Asparagine at location 126, which is located outside epitope regions. The change was detected as singular in three cases, and in one case it was found in combination with the previously presented epitope region 7-2 amino acid substitution at position 145.

Third subsequent sequence was available only in 11 cases, of which in five (45.5 %) cases, mutations were noted in comparison to the reference strain. In two cases,

the common amino acid mutation located at location 145 persisted from the second sample sequences, and in one case it was detected for the first time. Additional amino acid substitutions were also detected in those two cases that were found with the persisting amino acid mutation at location 145. These substitutions were at position 96 (Glycine to Serine) in epitope 7-1a and at position 211 (Asparagine to Aspartic acid) in epitope 7-1b. All presented amino acid substitutions located in epitope regions, with the exception of position 129 in region 7-1a, have been shown to escape neutralization with monoclonal antibodies. This finding may provide further understanding to effectors of long-term shedding after vaccination.

Based on this finding, the VP7 G1 sequences of the children reported in Study I were also studied. Of the 30 children, 28 were detected with G1 in stools, and in 14 of those children, the duration of shedding was longer than 14 days. Sequences covering the particular site were available in 10 cases. The same transition from G to A causing the amino acid change was found in 5 out 10 (50.0 %) sequences. All of these five children had received a minimum of two doses of the vaccine and at least 28 days before sampling. The same children were detected with G1 VP7 with vaccine VP6, whereas VP4 was negative in every case.

Analysis of P[8] VP8 and VP6 sequences*

A positive P[8] VP8* sequence was available from 21 of the first and nine of the second stool samples. In one of the first sample sequences, a nucleotide point-mutation was detected. The mutation caused an amino acid change (Phenylalanine to Tyrosine) at position 203 that located outside the epitope regions. Another nucleotide level change was noted but it did not cause changes at the amino acid level.

In the sequences of the second samples, only one nucleotide substitution was found: a transversion from G to T, causing an amino acid change from Valine to Leucine at position 163, outside the epitope regions. Although all VP8* amino acid changes were outside the epitope regions, all of the changes occurred in samples in which epitope region amino acid changes were detected in VP7.

When comparing VP6 sequences to the reference RotaTeq VP6 strain, no changes were found regardless of the sampling point in Study II, indicating lower genetic pressure in VP6 sequences. In the Study I, one of the five children with an epitopically located amino acid change in VP7 was found with a transition of C to T, causing an amino acid change from Methionine to Isoleucine at locus 302, which is outside the epitope region. In two children, a transition of C to T was found at

amino acid locus 324 in the epitope region. This mutation however caused no changes in the amino acid sequence and has been reported as indigenous(381).

5.1.6 Symptoms associated with vaccine strain shedding (II)

Symptoms related to RotaTeq vaccine shedding were evaluated using a symptom diary filled by parents for seven days. Some of the diaries were improperly filled or never returned, and out of 284, 194 and 227 diaries received after the respective dose of the RotaTeq vaccine, 278, 182 and 224, respectively, were included in the analysis.

The occurrence of gastrointestinal symptoms decreased during the course of immunization as vomiting (at least one episode) was reported in 35.3 % (98 out of 278), 34.6 % (63 out of 182) and 30.3 % (68 out of 224) children, after the respective vaccine doses (Table 9). Similarly, diarrhea (at least four stools per day) was recorded in 34.9 % (97 out of 278) children after receiving the first dose of the vaccine, in 30.2 % (55 out of 182) after the second dose, and in 15.6 % (35 out of 224) after the third vaccine dose. The proportion of children having long lasting diarrhea decreased throughout the vaccination course. By contrast, the proportional occurrence of fever (≥38.0°C) increased after the later doses, which were received concomitantly with other vaccines. Only 1.1 % (3 out of 278) of the children had fever after the first dose, whereas 3.3 % (6 out of 182) and 10.7 % (24 out of 224) of the recipients reported a temperature greater than 38.0°C after the second and third dose, respectively.

Of the 24 children with fever after the third vaccine dose, only six also had diarrhea and vomiting, and in two of these cases, the clinical severity according to the Vesikari score was severe. Interestingly, only one of these six children was detected with RotaTeq vaccine virus in stools just before the third dose, whereas the others did not (in one case, the second stool sample was not received).

The clinical severity of symptoms related to vaccination was evaluated by the Vesikari score. Similar to gastrointestinal symptoms, the clinical picture became milder after receiving more doses of the vaccine as the average of total score decreased from 5.3 after the first dose to 4.4 after the third dose of the vaccine. The median Vesikari score decreased from 5.0 to 4.0, respectively.

Table 9. Reported symptoms after each dose of the RotaTeq vaccine for seven days graded according to the Vesikari Score.

Symptom	Vesikari Score point	Diary		
		1st (N=278) *n (%)*	2nd (N=182) *n (%)*	3rd (N=224) *n (%)*
Duration of diarrhea (d)				
0	0	54 (19.4)	42 (23.1)	67 (29.9)
1-4	1	78 (28.1)	57 (31.3)	84 (37.5)
5	2	19 (6.8)	14 (7.7)	16 (7.1)
≥6	3	127 (45.7)	69 (37.9)	57 (25.4)
Max no. diarrheal stool/24h				
0	0	54 (19.4)	42 (23.1)	67 (29.9)
1-3	1	127 (45.7)	86 (47.3)	122 (54.5)
4-5	2	61 (21.9)	39 (21.4)	28 (12.5)
≥6	3	36 (12.9)	15 (8.2)	7 (3.1)
Duration of vomiting (d)				
0	0	180 (64.7)	119 (65.4)	154 (68.8)
1	1	25 (9.0)	19 (10.4)	19 (8.5)
2	2	20 (7.2)	9 (4.9)	15 (6.7)
≥3	3	53 (19.1)	35 (19.2)	36 (16.1)
Max no. vomiting episodes/24h				
0	0	180 (64.7)	119 (65.4)	156 (69.6)
1	1	37 (13.3)	29 (15.9)	32 (14.3)
2-4	2	44 (15.8)	29 (15.9)	26 (11.6)
≥5	3	17 (6.1)	5 (2.7)	10 (4.5)
Fever (°C)				
<37.0	0	45 (16.2)	19 (10.4)	26 (11.6)
37.1-38.4	1	232 (83.4)	160 (87.9)	181 (80.8)
38.5-38.9	2	1 (0.4)	2 (1.1)	12 (5.4)
≥39.0	3	-	1 (0.5)	5 (2.2)

Symptoms in relation to duration of shedding

Individual symptoms and the total clinical severity was compared in children with short- and long-term (RV positive stool sample before the third dose of the vaccine) shedding. No difference between individual symptoms and duration of shedding was detected after any dose of the RotaTeq vaccine (Supplementary Table 2 in Study II). However, long-term shedders were found with more severe symptoms after receiving the first dose of the RotaTeq vaccine, as of the 50 children with long-term shedding, 8 (16.0 %) had severe (Vesikari Score ≥11) and 12 (24.0 %) had moderate

(Vesikari Score 7-10) clinical severity whereas short-term shedders (N=172) the respective numbers were 10 (5.8 %) and 41 (23.8 %), respectively. The median (5.5 vs 5.0, respectively) and mean (5.7 vs 4.9, respectively) Vesikari Scores were also higher among the long-term shedders compared to the short-term shedders ($p=0.043$, χ^2 exact). No differences were found after the later doses (data not shown).

Symptoms related to shedding of vaccine-derived double-reassortant G1P[8]

The theorized higher virulence of vdG1P[8] genotype(357–359) was studied by comparing symptoms reported in cases where vdG1P[8] was found as the only genotype to cases with other genotypes, excluding combinations containing G1P[8] such as G1+G6+P[8]. In relation to the shed genotype, no difference in the total severity was found after the first dose of the RotaTeq vaccine. Of the 58 children detected with vdG1P[8], five (8.6 %) had a severe and 14 (21.9 %) a moderate clinical picture according to the Vesikari Score, whereas in children detected with other genotypes (N=198) the respective numbers were 15 (24.1 %) and 51 (25.8 %). While examining individual symptoms, no difference between the groups were found after any of the vaccine doses (Supplementary Table 1 in Study II).

5.2 Rotavirus epidemiology in Finland 4 to 9 years after vaccine introduction (III, IV)

Study III consisted of the first two years of the national RV follow-up from September 2013 to August 2015, and Study IV extended the surveillance for three more seasons up to August 2018. During this period, a total of 1,160 laboratory-diagnosed RV positive samples were received, and of those, 1,024 (88.3 %) were RT-PCR positive. Of the 1,024 RV cases, 837 (81.7 %) were detected in children, 55 (5.4 %) in adults and 132 (12.9 %) in the elderly.

RV seasonality

In children, the number of RV cases detected per season remained stable during the first four seasons of follow-up, but during the last season (2017-18) the activity was by far higher (N=259) (Fig. 7). No distinct pattern such as biennial occurrence was detected. In addition, the duration of the RV seasons remained stable, typically

beginning in January to March and ending in May to June, and no shift or prolongation of the season was detected.

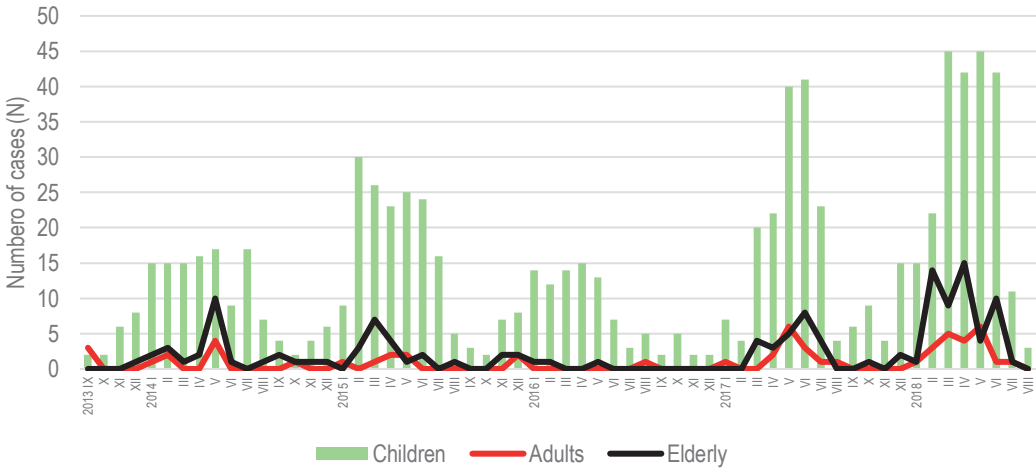

Figure 7. RV seasonality in children (N=837), adults (N=55) and the elderly (N=132) during five-season follow-up from September 2013 to August 2018.

In adults and the elderly, RV disease occurred more or less sporadically throughout the year for the first three seasons of follow-up. However, during the last two seasons, the occurrence of RV cases in both age groups followed a similar pattern to that of the children, with clearly higher activity during the spring months.

5.2.1 Circulating rotavirus genotypes in children (III, IV)

In total, G12P[8] was the most common genotype detected in children during the follow-up period from September 2013 to August 2018 (Fig. 8). The genotype was found in 195 (23.3 %) out of the 837 RV cases. However, it was mainly detected in the last season of surveillance (N=132). Of the other genotypes, G9P[8] (N=150), G1P[8] (N=128) and G2P[8] (N=105) were also typical findings in children. Other frequent detections were equine-like G3P[8] (N=68), G3P[8] (N=53), G9P[4] (N=50) and G4P[8] (N=43).

Several previously unseen and partly zoonotic genotypes were found occasionally or as single detections (N=11). The most common ones were G8P[14] (N=3) and G29P[9] (N=2) followed by others such as G2P[8] and G4P[6]. Mixed infections

were identified in 11 children, most of them associated with G-type G3: G3+G12P[8] (N=5), G3+G9P[8] (N=4), G3+G8P[8] (N=2) and G4+G9P[8] (N=1).

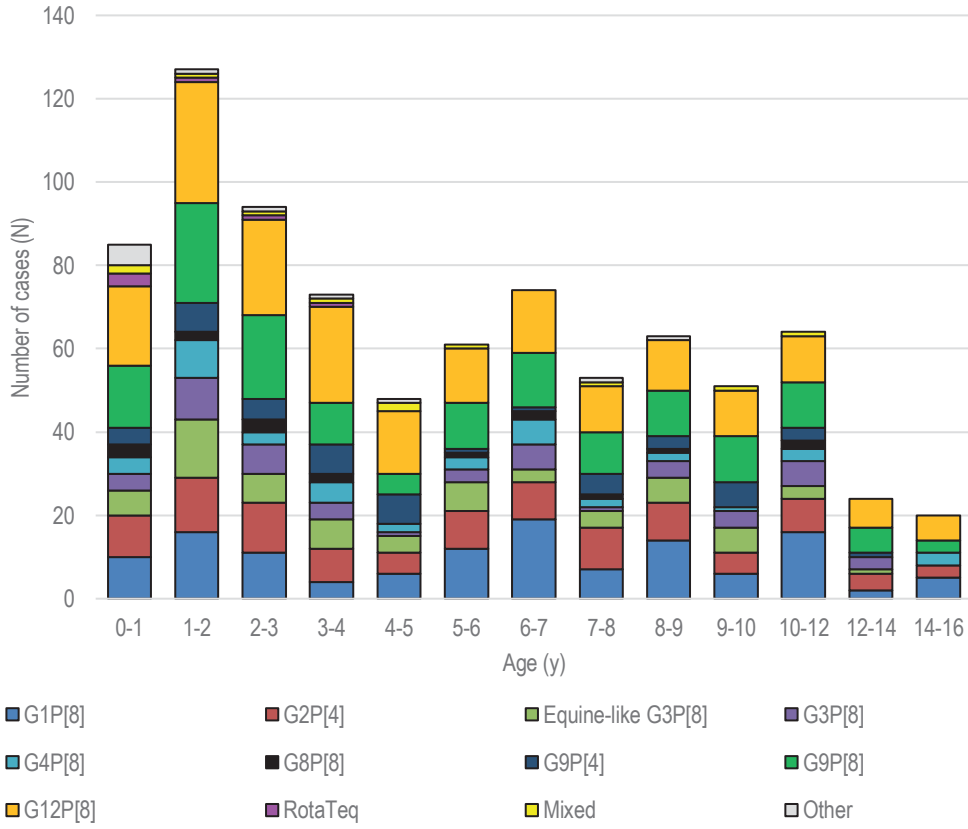

Figure 8. RV genotype distribution in children (N=837) by age from 2013 to 2018. RotaTeq includes all combinations including vaccine strain.

5.2.1.1 Rotavirus vaccine effects on rotavirus disease and circulating genotypes (IV)

Age distribution

The mean age for all 837 children was five years and the median 4.3 years, with a range from 8 days to 15 years. During the follow-up, the mean age of the children remained stable, from 4.9 years (2013-14) to 4.8 years (2017-18). However, a decrease in the median age was noted towards the end of surveillance, as the median age was 5 years in 2013-14 and 3.8 years in 2017-18.

Changes in the circulating genotypes

In the first season of follow-up, 2013-14 (N=129), G1P[8] was the predominant genotype with 44 (34.1 %) detections accompanied by G2P[4] (N=26, 20.2 %) and G4P[8] (N=24, 18.6 %) (Fig. 9). However, the proportion of these previously common genotypes decreased during the follow-up, as they formed together only 18 (6.9 %) out of the 259 cases detected in total during 2017-18. Correspondingly, the proportion of G12P[8] and G9P[8] increased, and a novel strain, G9P[4], appeared and became common. An equine-like G3P[8] was also detected for the first time during the 2014-15 season, and it soon became more common than the wild-type G3P[8]. A change in the predominant genotype was noted as the long-standing dominant genotype G1P[8] was first replaced by G9P[8] in the 2015-16 season, and G12P[8] became predominant from 2016-17 season.

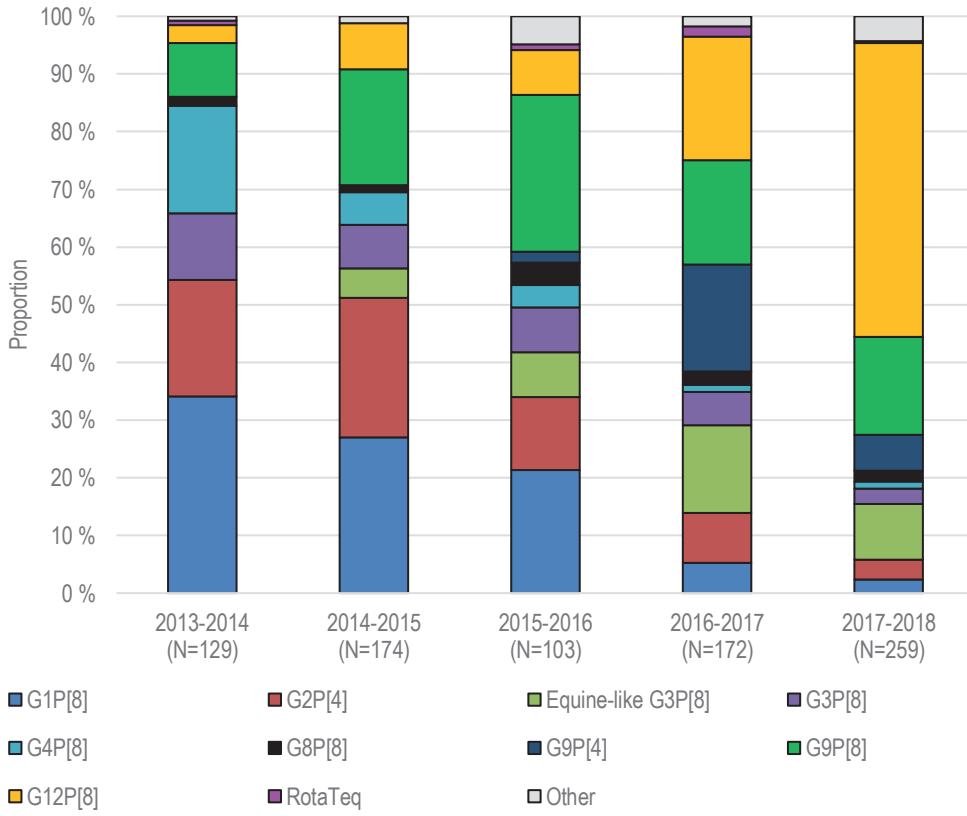

Figure 9. Prevalence of RV genotypes in children during each season from 2013 to 2018.

If a child was too old to receive the RotaTeq vaccine in the NIP (aged over two months in September 2009) and the vaccination status was unknown, the child was considered unvaccinated. This assumption was done to study the possible indirect vaccine effects on the cohort of older children aged four years and above. The proportion of these older children with RV (N=282) of all the children diagnosed with RV (N=837) decreased throughout the study period from 50 % (65/129) in 2013-14 to 20 % (52/258) in 2017-18. This decrease in the number of older children is in line with the decrease of the median age of children detected with RV, as described in the previous chapter. The changes in the circulating genotypes in this age cohort were similar to all children with the decrease of G1P[8] and G2P[4] and the increase of G9P[8] and G12P[8].

5.2.1.2 RotaTeq vaccine-derived strains (III, IV)

In Study IV, RotaTeq vaccine-derived viruses were detected in six children. However, the vaccination status was known in only one case of a recently vaccinated two-month-old child, who was detected with RotaTeq G1P[5] in the stools. Nevertheless, it is legitimate to assume that also two other children, aged three and six months and detected with RotaTeq G1+G4+P[8] and RotaTeq G1 VP7 only, respectively, had also received a dose of the vaccine lately. In two other cases, a possible transmission of vdG1P[8] was seen in children aged one and three years. A three-year-old was also detected with a mixed infection of wild-type G3P[8] in combination with RotaTeq G1 VP7.

5.2.1.3 Rotavirus in vaccinated and unvaccinated children (IV)

Vaccination status was reported in only 276 of the 837 RV cases in children. Of these 276 children, 172 were young enough to receive the vaccine in the NIP, and of these children, 96 (55.5 %) were known to have received at least one dose of the RotaTeq vaccine and 76 children were unvaccinated. In addition, 13 children had received RV vaccine before the beginning of mass-vaccinations; these children were not included in the following analyses.

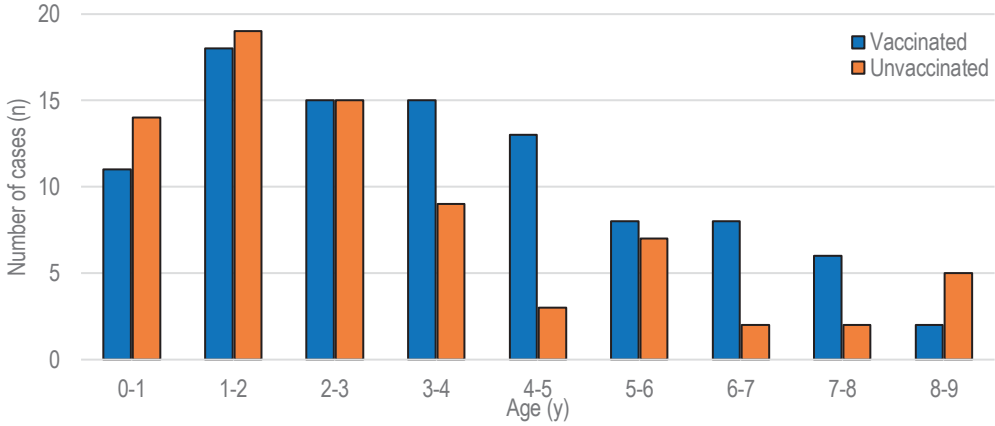

Figure 10. Age distribution of vaccinated (N=96) and unvaccinated (N=76) children with a RV infection. Figure reproduced and adapted with the permission of the publisher, Elsevier(382).

Interestingly, both the mean and median age of vaccinated children (3 and 2.7 years, respectively) were higher than that of the unvaccinated children (2.7 and 2 years, respectively). This difference was statistically significant (p=0.05, Mann-Whitney U test) (Fig. 10). The genotype distribution was otherwise even but G12P[8] was a considerably more common cause of RV disease in vaccinated than unvaccinated children with 38 versus 13 detections, respectively (p=0.001, χ^2 test). By contrast, G4P[8] was more common in unvaccinated (N=11) than in vaccinated (N=4) children (p=0.017, χ^2 test) (Fig. 11).

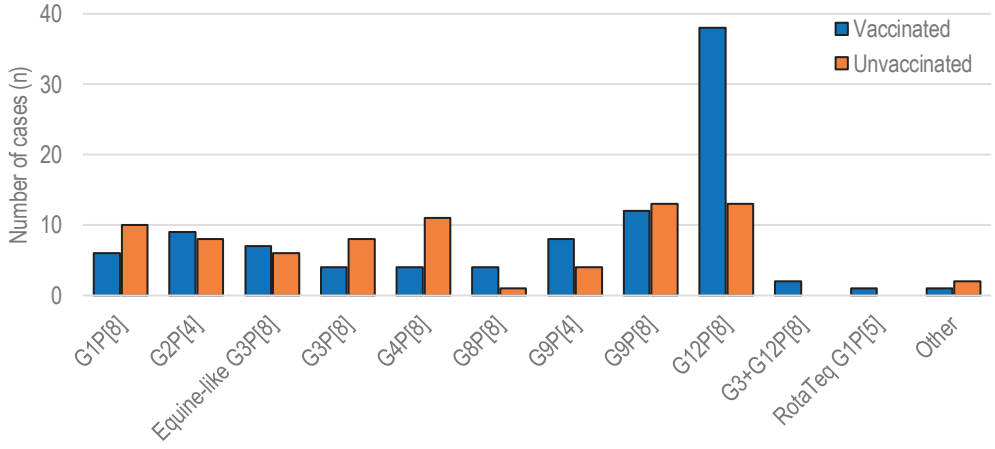

Figure 11. Rotavirus genotype distribution of vaccinated (N=96) and unvaccinated (N=76) children. Figure reproduced and adapted with the permission of the publisher, Elsevier(382).

5.2.2 Rotaviruses detected in adults and the elderly (III, IV)

In adults aged 16 to 69 years, total of 55 cases of RV were confirmed between September 2013 and August 2018. The mean and median ages were 38 and 41 years, respectively. Most of the cases were caused by G2P[4] (N=17, 30.9 %) followed by G9P[8] (N=7, 12.7 %) and G12P[8] (N=7, 12.7 %) (Fig. 12). Several zoonotic strains, such as G29P[9], G3P[6] and G6P[14], were also identified. Mixed infections (three cases caused by G3+G9P[8] and one case by G2+G12P[4]) were found in adults, but not in the elderly.

The number of RV cases was more frequent in the elderly aged 70 years and above, where RV was detected in the stool samples of 132 cases. In this age group, the mean age was 84 years and the median age 85 years. The genotype distribution was similar to that for the adults; G2P[4] (N=50, 37.9 %) predominated and G12P[8] (N=30, 22.7 %) and equine-like G3P[8] (N=12, 9.1 %) were the second most common genotypes. In the elderly, G8P[14] was the only zoonotic strain detected.

During the follow-up period, some changes in the circulating genotypes were noted in both age groups as G2P[4] dominated at the beginning of surveillance but was then replaced by G12P[8] during the last season of 2017-18. Actually, in addition to G12P[8], both G9P[4] and G9P[8] became common first in children and a season later in adults and the elderly.

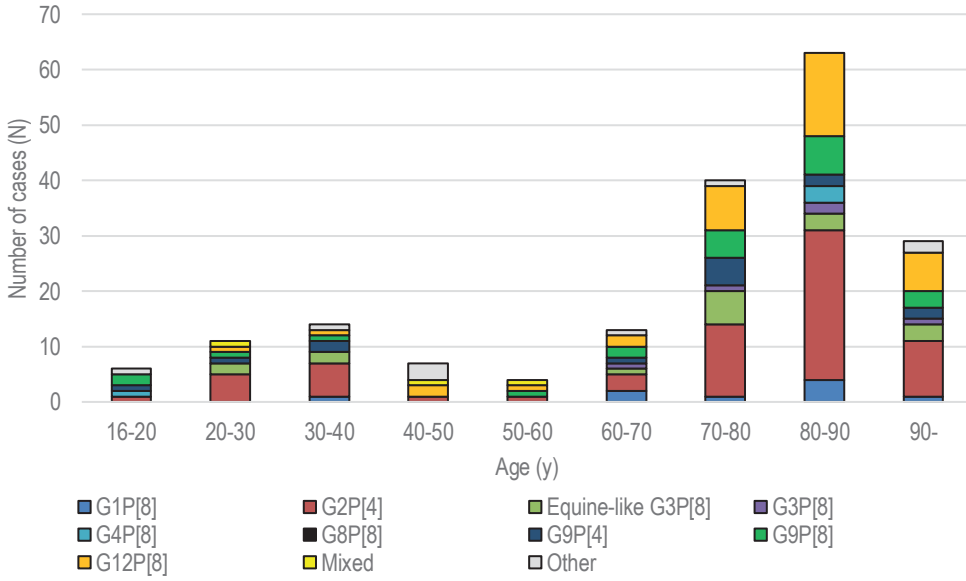

Figure 12. RV genotypes by age in adults (N=55) and the elderly (N=132) during the five seasons of follow-up 2013-2018.

6 DISCUSSION

6.1 Shedding of RotaTeq vaccine rotaviruses

The section 5.1, which contains results of Studies I and II together with some unpublished data, increased the knowledge regarding the characteristics of the shedding of RotaTeq vaccine RVs. Study I already showed that excretion of vaccine viruses into stools after inoculation was more common than previously shown in the pre-licensure studies(363). In some cases, the duration of asymptomatic shedding was surprisingly long, raising questions about the prolonged infection of the gut in immunocompetent infants. However, the study material was not fit for evaluating the true extent and duration of shedding, as it consisted of children hospitalized for RTI at a random time point, in relation to the last received RV vaccine dose.

The more systematic Study II showed that indeed almost all of the vaccine recipients excreted vaccine strains in the stools after receiving the first dose of the vaccine. This high rate is in line with findings of post-licensure studies by Hsieh et al.(367) and Ye et al.(368). Differing from the conducted study protocol, the intended original study design contained additional stool sampling immediately before and after the second vaccine dose accompanied by serum samples after each dose. Unfortunately, the study was forced to be re-designed to enhance recruitment, as the protocol was too burdensome for both the infants and parents. Logically, one might speculate that the rates would be even higher than those that were observed 2.5 months and 1.5 months after the second and third doses, respectively. This is based on the high proportion of children shedding after the first dose of the vaccine, and also on the findings made by earlier studies in which the rates of shedding were high after the second and third dose of the vaccine(367,368).

In Study I, half of the children were detected with prolonged shedding and a third continued to shed over a month after the last received dose of vaccine. However, in Study II, the number of shedders after the following doses decreased more rapidly than assumed. Only a fifth of children presented prolonged shedding between the first and the third dose, and a fifth of those continued shedding even after the third dose. A likely reason for the lower rates was the previously mentioned longer time from inoculation to sampling excluding shorter shedding. Still, in a few cases

shedding continued for four to 5.5 months in total. One of our original theories was that the long presence of the vaccine RVs in the gut of these children was possibly due to a lack of seroconversion or IgA. However, the great majority of the children in fact seroconverted against VP6 IgA suggesting that despite the extensively long asymptomatic shedding, these children had obtained protection from vaccination. Even though the number of samples was low, the seroconversion rate was similar to what has been previously reported(160,383).

Genotyping of shed vaccine strains in children hospitalized for RTI without AGE symptoms was conducted to our knowledge for the first time in Study I. Other studies(366–368) have focused mostly on detecting vaccine viruses without classifying them, with the exception of a vaccine development study by Clark et al.(275) and a recent real-time RT-PCR methodology study by Higashimoto et al.(369). Compared to those, the predominance of G1 was surprising in both Studies I and II, and the strain was practically the only one associated with prolonged shedding. This suggests that despite containing several different RV strains, the RotaTeq vaccine might actually work *in vivo* in a similar manner as the single G1P[8] strain vaccine, Rotarix. This is supported by the fact that despite the difference in the number of RV strains between the vaccines, the efficacy is approximately the same against different genotypes(160,161,168,277). The backbone of the vaccine consists of the WC3 bovine strain, which as a vaccine candidate was shown to elicit insufficient protection in humans but was tolerated well(235,236). Reassortment with different human VP7 and VP4 proteins increased the immunogenicity of the vaccine to the proper level(384). In many cases, the human origin G1 VP7 and P[8] VP4 proteins were detected together, suggesting re-reassortment, which could be due to a structural preference, as these proteins in fact originate from the same G1P[8] RV strain and therefore they might combine more eagerly with each other than with the artificial bovine components. This theory might be argued as a similar re-reassortment of G3 and G4 with P[8] was minimal in the current material, while both of these VP7s originated from parent strains that contained P[8](271).

On the basis of previous studies(357–359), vdG1P[8] was expected to be more virulent than the original vaccine strains, but the severity of the individual symptoms and the clinical picture by the Vesikari score of the children did not differ regardless of the genotype shed. In addition, several children in Study I were detected with vdG1P[8] and still were hospitalized due to RTI without AGE symptoms. Overall, the combination was common in both of the studies, suggesting the possibility that the newly formed vdG1P[8] would continue to infect other enterocytes and replicate

at an even higher rate compared to the original vaccine strains. In fact, in a few cases the viability of the strain was successfully proved in cell culture.

As discussed earlier in section 2.4.6, vdG1P[8] was originally noticed already in the studies of the quadrivalent version of the current RotaTeq vaccine. In fact, Ward et al.(275) even studied neutralizing antibody responses against vdG1P[8]. The strain showed similar rates of seroconversion as the sole G1, but the geometric mean titers were higher compared to other vaccine components, suggesting that vdG1P[8] could be immunologically important. After combining this information with the current findings of the strain being the most commonly shed combination in a real-life setting, despite not even being a part of the composition, the main mechanism of the function of the RotaTeq vaccine might be this recombination. If so, the two vaccines – RotaTeq, the five-strain-containing human-bovine reassortant vaccine, and Rotarix, the single human G1P[8] strain vaccine – may work similarly. This theory is supported by the absence of difference between the real-life effectiveness of the two vaccines in post-licensure studies(301).

Another goal was to assess symptoms related to the duration of shedding, but no single symptom was found to relate to prolonged shedding. However, when assessing the clinical picture by using the Vesikari score, children who became prolonged shedders had a higher total score after the first dose of the vaccine. It may be speculated that for a still unknown reason, these individuals may somehow be more susceptible to RV in comparison to short-term shedders. The symptoms reported by the parents differed from those reported by the RotaTeq safety studies. In the present material, gastrointestinal symptoms were found far more often than previously reported, but this was possibly due to the over-reporting of newborns' stools and spit-ups by the parents.(160) As it was not the original intention, the study did not include a placebo group that would at least partially eliminate such an over-reporting bias. Interestingly, in comparison to the safety studies, fever was seen more seldom in the current material, even though concomitant vaccinations were also given in the safety studies. This positive effect has no good explanation; it is possible that the concomitant vaccines currently in use in Finland are less reactogenic than the ones used during the pre-licensure studies. Overall, the RotaTeq vaccine was well tolerated but in some individuals, severe symptoms related to the vaccination may predict the prolonged duration of shedding.

The viability of the shed G1-containing strains was assessed by cultivating a selected subset of samples in MA104 cells. The selection was guided by the both RT-PCR and ELISA results, and as assumed, a positive ELISA and a positive RT-PCR for all three capsid proteins were required for successful multiplication. It seems that

prolonged shedding was associated with the shedding of proteins rather than whole viral particles, as the samples obtained before and after receiving the third dose of the vaccine showed to be cell culture negative. Nonetheless, how only particles of viruses and not the whole viruses are preserved in the gut for months without active replication is a mystery. Gut microbiota might have a role, as in a study on the effect of the microbiome on the immunogenicity of Rotarix in Indian children, it was noted that the bacterial microbiome differed in relation to shedding status and that the shedders were found with more enteroviruses at the time of vaccination(385).

Investigation of the sequences of the most commonly shed genotype, G1 revealed a consistent change in the amino acid sequence of the majority of the second samples obtained from children with prolonged shedding. The mutation was located in the epitope region of VP7, and the locus is known to cause escape from neutralizing mAbs(334). Inspired by this finding, the randomly collected sample material of Study I was re-examined and the same single-point mutation was found in lower scale. Likewise, in all of those cases, shedding had continued for longer than 14 days and the child had received at least two doses of the vaccine, similar to unpublished genetic data from the Study II material. Even though in the majority of cases there was only a one amino acid change, previous studies have shown that such a small change may a have large effect on neutralization overall. It is possible that this impairment could lead to a broader or more intense infection of the intestinal mucosa, which could then be seen in more severe clinical symptoms. However, the amino acid changes were not detected until after receiving the second dose of the vaccine, while the children with prolonged shedding were reported to have more severe symptoms after the first dose. Due to the sparse sampling between the first and the third dose, it is nevertheless possible that the change occurred already after the first dose.

On the basis of these findings, it was surprising that during the five RV seasons reported in Studies III and IV, only a total of six children were found with the RotaTeq vaccine strain in stools. The number is much lower than what has been reported for example in England, where over 200 cases of the Rotarix G1P[8] strain were detected during three RV seasons with a ten-fold larger population(362). As the rates of shedding for both of the vaccines are roughly the same, one explanation might be differences in testing practices or the higher proportion of children in relation to the overall population(367).

Nonetheless, in two cases, children aged one and three years, transmission of vdG1P[8] from a vaccinated contact was likely. Unfortunately, the vaccination status of the children was not available, but they would have been eligible to receive the

vaccine according to their age. These and the previously reported cases of symptomatic transmission of vdG1P[8] have been seen in children aged one year and above(357,359,386). The older age of these children may be a manifestation of immunological waning, as it could be assumed that these children would have received the RV vaccination and/or encountered RV beforehand, and therefore it would have elicited at least some level of protection. On the other hand, it is possible that, in contradiction to the current findings, vdG1P[8] indeed has a higher virulence, and, in combination with waning immunity, could lead to a symptomatic infection.

From a clinical point of view, according to the data presented by this thesis, shedding of the RotaTeq vaccine strains is common but not harmful to a healthy individual, regardless of the duration. The long duration seems not to have a negative effect on protection, at least when studied by using seroconversion for serum VP6 IgA. On the other hand, the development of difficult prolonged diarrhea has been reported in children with impaired immunity – such as undiagnosed severe combined immunodeficiency – who receive a live RV vaccine, development of difficult prolonged diarrhea have been reported(370,372). Another increasing population with potentially impaired immunity are the infants whose mothers are treated with tumor necrosis factor-alpha-antagonists during pregnancy. These drugs are IgG monoclonal antibodies that have been shown to actively transfer transplacentally, and measurable levels can be found even up to seven months after birth(387). The subject needs further investigation as there are no studies on whether these drugs have an affect on RV vaccination or not. In addition, viable vaccine viruses are shed for at least 10 days after inoculation, which in turn may form a threat to immunocompromised contacts of all ages. On the other hand, a more likely and beneficial scenario would be transmission of these vaccine strains to immunocompetent unvaccinated individuals eliciting protection without actual vaccination.

6.2 Epidemiology of rotavirus in Finland 4 to 9 years after vaccine introduction

During the five years of follow-up covering the fourth to the ninth year since the introduction of the RotaTeq vaccine in September 2009, the burden of RV in Finland has remained stable. A natural season-to-season fluctuation was detected, including higher RV activity in 2017-18 season. Despite the higher number of total RV cases during the particular season, the number of cases remained lower than in

the pre-NIP period, with the numbers indicating that the RotaTeq vaccine has maintained its high efficacy(201). Similar sustained low activity after introduction of the RV vaccine has been reported in other high vaccine coverage countries such as Belgium, the US and also the low-income country of Ghana(300,303,388).

After the introduction of the RV vaccine, most of the previous studies have detected a shift of the disease to unvaccinated older children(300,389–391). On the contrary, the current findings suggest the opposite, as the age structure of the children was younger at the end of surveillance when compared to the first season. Similarly, following the robust approach of dividing children into the vaccine-eligible and -ineligible according to age, a consistent decline was seen in the number of RV cases in the older vaccine-ineligible children. A similar long-term tendency has been reported in Belgium also, which has a similar high-coverage setting with the Rotarix vaccine(300). This trend is probably due to the increasing number of vaccinated age cohorts, but also due to herd protection elicited by the vaccine. It is possible that a similar shift of the disease to older age groups, as reported by others, has also occurred in Finland during the first years after the vaccine introduction, but due to continuous mass vaccination at a high vaccination coverage, the cohort of unvaccinated older children has decreased constantly.

RV strains have always fluctuated at some level, but changes in the predominant strains are more seldom. Current time frame contained the special turnover of strains, as the traditional G2P[4], G3P[8], G4P[8] and especially G1P[8] diminished and were replaced by the novel G9P[8], G9P[4] and G12P[8]. The waning of G1P[8], a strain that has been predominant in Finland almost uninterruptedly since the 1980s, was an important finding(200). The replacement of G1P[8] has been seen in many countries regardless of the vaccine used, but usually this change has been more rapid. The currently depicted genotype distribution in Finland resembles those reported in the US and Australian RotaTeq-using territories, and in turn it differs from the Rotarix-using countries, suggesting that RV mass vaccination might indeed have an effect on circulating strains.(316,324,326,362)

Despite the changes in the circulating genotypes and the emergence of new strains such as G9P[4] and equine-like G3P[8], no drastic changes in the number of RV cases were detected. Even though the latest season of the follow-up seemed to include an outbreak caused by G12P[8], the vaccine efficacy against the strain was in fact approximately 85 % regardless of the number of received doses of RotaTeq (Tuija Leino, THL, personal communication, November 2018). Even though the information regarding vaccination status was available for only some of the children, G12P[8] was significantly more prevalent in the vaccinated compared to the

unvaccinated children. In the US, Ogden et al.(330) have reported the high prevalence of G12P[8] in vaccinated children, noting also that the strain differed significantly from the RotaTeq vaccine strains causing antigenic mismatch, potentially explaining the result being similar to ours. The increased diversity of detected RV genotypes may be partly due to the development of better detection methods such as RT-PCR and sequencing over RV antigen ELISA. However, other factors may have an influence as well, as the mobility of humans has increased in recent decades, enabling the more efficient spread of strains to new locations and to possibly more immunologically susceptible areas. Despite the increased diversity, the sustained low activity supports previous studies, suggesting that RotaTeq vaccine elicits broad heterotypic protection, and RotaTeq has been shown to be efficacious against these emerging strains(160,277,392,393).

The study material consisted of all laboratory-confirmed RV cases covering all age groups, and therefore enabled to study RV disease in adults and the elderly. After the first two years of surveillance, as reported in Study I, it seemed that the RV disease in these age groups was significantly different from the disease seen in children. RV cases occurred evenly throughout the year without distinctive higher activity months and the genotypes were different. During the two first study seasons, G1P[8] continued to predominate and G9P[8] became common in children, whereas in adults G2P[4] was by far the most common genotype. These differences raised a theory that RV disease in children and adults could in fact be their own entities, and that the adults possibly function as a reservoir for the disease of the children. In fact, some of the previous studies have reported a lack of association between child contact and RV disease in adults who were hospitalized for RV(207,214).

Extension of the surveillance time by another three seasons clarified the connection between children and adults. The disease in adults and the elderly began to follow the seasonal changes seen in children more clearly, with higher activity during the winter months. Previous studies have made the same finding(207,214,394). Contradicting the earlier theory, it seemed that the disease was in fact transmitted from children to older age groups, as the emergence of genotypes such as G9P[4], G9P[8] and G12P[8] was noticed a season before in children. This children-to-adult transmission was suggested already in the 1980s, and it is likewise supported by more recent studies reporting a decrease in the number of RV cases in the adult population after the vaccine introduction in children(204,210,394). However, such a decline was not observed probably because the surveillance was dated four to nine years after vaccine introduction and it contained no data before or immediately after the implementation of the vaccine. Overall, the number of RV

cases in adults (16 to 65 years of age) was small, whereas according to this thesis RV might be an underestimated pathogen in the elderly. Unfortunately, patient record were inaccessible to determine the possible co-morbidities that may predispose to RV infection(207–209).

7 CONCLUSION AND FUTURE PROSPECTS

In 1980, Kapikian et al.(228) stated: "Before an effective rotavirus vaccine can be formulated, we must understand the mechanisms of host resistance to rotavirus infection". Approximately 40 years later, we still do not know the exact mechanisms for RV immunity, nor the correlates of protection. However, we have instead witnessed the success-story of live-saving vaccines whose development was ultimately based on the Jennerian approach, like the smallpox vaccine. Despite the fact that RV vaccines have saved millions of lives and averted even higher number of hospitalizations, the basic underlying biology is still in many parts unknown.

This thesis characterized the shedding of the live human-bovine reassortant RV vaccine RotaTeq that is currently used in Finland with a high vaccine coverage. Excretion of vaccine strains in the stools of recipients is common, and the duration may vary greatly from days to prolonged shedding continuing up to months, even in healthy asymptomatic children. The reason for the difference in the duration between individuals remains unknown and needs further investigation. A consistent amino acid mutation causing changes in the epitope region of the outer layer protein VP7 was found. This may cause escape of the virus from neutralizing antibodies and thereby explain the prolonged presence of the vaccine strain in the gut. In addition, more severe symptoms after the first dose may be an indication of the beginning of prolonged shedding. However, in the light of the current study, viable vaccine viruses are excreted into stools for only a short period after inoculation, whereas prolonged shedding consists of non-infectious particles of these viruses. From a clinical perspective, such information is important and close contacts between recently vaccinated infants and immunocompromised individuals should be avoided. On the other hand, the spread of vaccine viruses to unvaccinated toddlers would be beneficial, potentially eliciting protection in the unvaccinated recipient.

Other possible and even more important findings were the major association of outer capsid protein G1 with shedding and the common formation of a so-called vaccine-derived double-reassortant G1P[8] strain. The latter has been previously connected to higher virulence, but support for such a linkage was not found. Overall, the data suggests that in a real-life setting, the RotaTeq vaccine containing five different reassortant RVs functions in a similar manner as the single G1P[8] strain

vaccine, Rotarix. This finding would well explain the almost similar effectiveness of these two vaccines, highlighting the importance of cross-protection despite the difference in the number of strains contained.

The effects of mass vaccination on circulating RV strains has been discussed since the introduction of RV vaccines due to the fear of potential vaccine-related selection pressure leading to lower vaccine efficacy. Regardless of the lack of exact knowledge on the method of function of the RotaTeq vaccine, on national level in Finland, the vaccine has remained highly effective for nearly a decade after implementation. Mass vaccination at a high vaccine coverage has stabilized RV activity to a low level with minor natural fluctuation between the seasons. On the other hand, this five-season follow-up took place on the verge of a major shift of circulating genotypes in children, as the traditional strains; G1P[8], G2P[4] and G4P[8] were superseded by the emerging G12P[8], G9P[4] and G9P[8]. This phenomenon has been reported in other RotaTeq-using countries, such as the US and Australia, and it indeed may be a reflection of vaccine pressure. In addition to the turnover detected in the circulating genotypes, broadening of the strain spectrum was identified in all age groups. These novel strains have mainly occurred as rare detections but genotypes such as equine-like G3P[8] have spread globally and largely superseded the human originated G3P[8]. Nevertheless, these strains have not become common, supporting the heterotypic protection elicited by the current RV vaccines.

RV samples were also received from adults and the elderly. These age groups were under keen interest during the first decade of the RV studies, but they have since been somewhat forgotten. The burden of disease in adults seems low but in the elderly, RV may in fact be a clinically important but underestimated cause of AGE. Unfortunately, despite RV disease in adults has been highly associated with other co-morbidities, the current material did not contain clinical data and studying co-morbidities was not possible. Yet, it is reasonable to assume that these cases were from hospitalized children and adults.

Although RV disease in children seemed stable, important vaccination-related changes were detected. The number of children who were too old to receive the vaccine at the time of the introduction in September 2009 decreased consistently. Additionally, the age of diseased vaccinated children has exceeded the age of unvaccinated children. Both findings indicate the effects of herd immunity related to a near decade of high-coverage vaccinations. After all, the fact that RV continues to circulate, indicates that the current live oral RV vaccines are not able to eradicate the pathogen despite almost optimal conditions. Current vaccine development has taken the course towards non-live particle vaccines, potentially eliminating the few

imperfections that the current vaccines have – i.e. potential vaccine-related selection pressure, intussusception and lower real-life efficacy in high mortality settings. Only time will tell if the development of such vaccines will continue the success story of RV vaccines seen up to date. Nonetheless, one thing remains certain: Diarrhea is moist poo.

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Jukka Markkula

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ORIGINAL COMMUNICATIONS

PUBLICATION I

Detection of vaccine-derived rotavirus strains in nonimmunocompromised children up to 3-6 months after RotaTeq vaccination

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Detection of Vaccine-derived Rotavirus Strains in Nonimmunocompromised Children up to 3–6 Months After RotaTeq® Vaccination

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Abstract: We conducted a survey on the presence of RotaTeq vaccine viruses in infants hospitalized with respiratory infection, and detected shedding in 17% of children (<2 years of age) who had ever received the vaccine. The latest detection was at the age of 8 months. We conclude that asymptomatic long-time shedding of RotaTeq viruses is not uncommon, and is particularly associated with genotype G1.

Key Words: RotaTeq, rotavirus, vaccination, shedding

(*Pediatr Infect Dis J* 2015;34:296–298)

RotaTeq® (Merck & Co., Whitehouse Station, NJ) is a live oral rotavirus (RV) vaccine consisting of 5 human-bovine reassortant vaccine viruses. In Finland, RotaTeq vaccine was taken into the national immunization program in September 2009, and is administered on a 3 dose schedule at the ages of 2, 3 and 5 months.

Shedding of RotaTeq vaccine viruses was reported low in the prelicensure trials,[1] but on a recent reanalysis of the Rotavirus Efficacy and Safety Trial (REST) study material using reverse transcription-polymerase chain reaction (RT-PCR), shedding of RotaTeq vaccine viruses was detected in up to 65% of the vaccinees with gastroenteritis symptoms.[2] Thus, the full extent of shedding of RotaTeq vaccine viruses is not yet characterized. We examined the occasional presence of RotaTeq vaccine viruses in young children seen in hospital mainly for respiratory tract infection (RTI) and correlated these findings with history of RV vaccination. This approach yielded new information on the extent, duration, and type of RotaTeq vaccine virus shedding.

MATERIALS AND METHODS

Clinical Methods

A prospective study approved by the Ethics Committee of Pirkanmaa Hospital District on the etiology of RTI in children was conducted at Tampere University Hospital from September 1, 2009 to August 31, 2011. All children under 15 years of age, who were admitted into pediatric ward with RTI were eligible for the study.

In this study a stool sample was collected during the hospitalization by a study nurse or nurses working in the pediatric ward or, if not successful, the parents were provided with a sample kit to send a stool specimen within 2 weeks from home. We used this material to examine the presence of rotaviruses and specifically RotaTeq vaccine viruses and therefore excluded all children who had not received any dose of RotaTeq. Dates of administration of RV vaccine brand as well as the vaccine used was enquired from the parents and confirmed from the records of the respective well baby clinic by a study nurse.

Laboratory Methods

Stool samples were stored in freezers at −70°C. Viral RNA was extracted using Qiagen QIAamp Viral RNA Mini Kit (Hilden, Germany) according to the manufacturer's instructions. RV viral proteins VP7, VP4 and VP6 PCRs, enzyme-linked immunosorbent assay (ELISA) test and sequencing of PCR positive samples were performed as previously reported.[3]

Positive stool samples were propagated in fetal rhesus monkey kidney (MA104) cells as described previously[3] with the modification of using minimum essential medium with 0.5 μg/mL of trypsin instead of minimum essential medium containing 100 U/mL penicillin, 100 U/mL L-glutamine and 100 μg/mL streptomycin.[4]

RESULTS

During the 2-year-study period a total of 944 children with RTI were recruited into the study and 557 (59.0%) children provided a stool sample. A total of these children, 182 (32.7%) had ever been vaccinated with RotaTeq at any time after September 1, 2009, thus forming our study population. The mean age of the study population was 256 days, ranging from 57 to 643 days; 73.1% were males.

Out of the 182 stool samples, 30 (16.5%) were RV positive by RT-PCR specific for VP7. RV positive samples were identified as RotaTeq vaccine type by sequence analysis. VP6 RT-PCR was positive in 29 and negative in 1 of the 30 VP7 positive cases. Bovine vaccine type VP6 was detected in 28 cases and human type VP6 in 1 case, described later. VP4 RT-PCR was positive in 19 and negative in 11 cases.

RV antigen by ELISA was tested from 19 of the 30 VP7 positive cases. ELISA could not be performed from the remaining 11 samples due to an insufficient amount or type of the sample (diaper). Out of those 19 samples, only 4 (16.7%) were ELISA positive. The ELISA positive cases were all detected with RotaTeq G1P[8] double-reassortant; 1 child was also detected with an additional P[5] VP4.

RotaTeq G1 sequence was detected in 28 cases (93.3%) out of 30 VP7 positive cases. The double-reassortant combination G1P[8] was detected in 11 children (36.7%). The original vaccine type G1 reassortant G1P[5] was detected in 4 children (13.3%), and 1 child had RotaTeq G1 with both P[5] and P[8]. Also 11 children (36.7%) were detected with RotaTeq G1 alone (with no VP4 sequence detected). VP4 reassortant G6P[8] was detected in 2 children. (Table 1)

One child was detected with human VP6 reassorted with RotaTeq G1 VP7 and human wild-type VP4 P[8]. The stool sample of this child was obtained 10 days after the second dose of RotaTeq vaccine. The sample was extracted for several times, and RT-PCR and sequencing were done twice for each protein from each extraction, but the RT-PCR and sequencing results remained identical. The sample could not be propagated in MA104 cells.

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TABLE 1. Relative Frequencies, % (n), of RotaTeq Vaccine-originated Rotavirus Genotypes and Their Combinations Detected After Each Dose of RotaTeq Vaccine in Children Hospitalized With Respiratory Infection

Genotype	1st Dose (n = 14)	2nd Dose (n = 10)	3rd Dose (n = 6)	Total (n = 30)
G1P[8]	57.1 (8)	20.0 (2)	16.7 (1)	36.7 (11)
G1P[5]	7.1 (1)	20.0 (2)	16.7 (1)	13.3 (4)
G1 + P[5] + P[8]	7.1 (1)	0	0	3.3 (1)
G1 + wild-type P[8]	0	10.0 (1)	0	3.3 (1)
G6P[8]	7.1 (1)	0	16.7 (1)	6.7 (2)
G1 alone*	21.4 (3)	50.0 (5)	50.0 (3)	36.7 (11)

*VP4 RT-PCR negative.

A total of the 182 RV vaccinated children, 28 had received 1 dose, 38 had received 2 doses and 116 had received all 3 doses of RotaTeq vaccine at the time they were hospitalized. Shedding of RotaTeq vaccine virus was detected in 14/28 (50.0%) stool samples collected after the first and before the second dose. After the second and before the third dose vaccine virus shedding was detected in 10/38 cases (26.3%), and after the third dose in 6 out of 116 cases (5.2%).

RotaTeq G1P[8] double-reassortant was commonly shed after each dose of the vaccine. After the first dose 8 children shed RotaTeq vaccine-derived G1P[8], and 3 children shed after the second and 1 child after the third dose. None of these children had diarrhea. The vaccine strains and combinations in relation to the latest vaccine dose are shown in Table 1.

The duration of shedding, as counted from the latest vaccination date, was over 14 days (prolonged) in 16 cases (53.3%) and over 30 days in 9 cases (30.0%). The proportion of long-time (over 14 days) shedders became larger after each dose of vaccine received; after the first dose prolonged shedding was detected in 4/14 (28.6%) children, respectively after the second 7/10 (70.0%) and after the third dose 5/6 (83.3%).

The longest duration of shedding was 84 days counted from the third immunization in a child detected with RotaTeq G1 VP7, while VP4 and VP6 RT-PCRs were negative. The other 5 cases after the third dose were detected 9, 22, 39, 52 or 53 days after the third dose, and the respective genotypes were RotaTeq G1 alone, G1P[5], G1 alone, G6P[8] and G1 alone.

DISCUSSION

In this study we used children hospitalized for respiratory infection without gastroenteritis symptoms as proxies for healthy children to follow the shedding of RotaTeq vaccine viruses after routine vaccination. In these children under the age of 2 years who had received RotaTeq vaccine we detected the presence of RotaTeq vaccine viruses in 16.5%. This is higher than detected in the prelicensure studies using the plaque assay method on samples collected within a week after each dose,[1,5,6] but largely in line with more recent studies, which have shown shedding rates of around 20% using RT-PCR or enzyme immunoassay as detection methods.[7–9]

The previous studies have not detected prolonged shedding on such a scale, although Hsieh et al[10] detected RotaTeq strains up to 28 days after inoculation. Very long shedding, like our finding up to the age of 8 months, has not been described previously in healthy infants, but Patel et al.[11] detected shedding over 200 days in immunocompromised children. We detected 6 children with prolonged shedding 9–84 days after the third dose of RotaTeq vaccine. However, it is not certain after which dose the shedding started in such cases. It is possible that our occasional sampling detected children who became long time shedders already after the first dose, in which case the longest duration of shedding might have been 6 months.

Dominance of RotaTeq G1 genotype in shedding was a new finding, as G1 was detected in 93.3% of the cases. Still, also in prelicensure studies of RotaTeq G1 and P[8] were actually the most common genotypes shed after vaccination, and also the G1P[8] combination was detected already in the early studies.[12,13] In the composition of RotaTeq vaccine, the titer of G1 is nearly the same as that of G3 and G4, and lower than G2; therefore the higher shedding rate of RotaTeq G1 cannot be explained by a higher inoculum.[14] In fact we did not detect any other vaccine-derived human G-types but G1 among the shedders. The only unifying factor with prolonged shedding was RotaTeq G1 VP7, with the exception of 2 cases with bovine–human G6P[8] combination. The properties that make G1 such a predominant genotype in prolonged infection, and consequently shedding, remain unknown and need further study.

The major limitation of our study was the design of the original study, which was not planned to determine the rate or duration of shedding of RotaTeq vaccine, but only provided stool samples at random time points. However, even with this less than optimal study design we could determine that long term shedding of vaccine viruses is not uncommon. While we could not determine after which dose the long-term shedding started, it is reasonable to speculate that already the first dose may select those individuals who eventually become long-term shedders.

Shedding of RV vaccine strains in asymptomatic children is usually not regarded as clinically significant, with the possible exception of transmission to susceptible or immunocompromised contacts. The use of sensitive RT-PCR in detection of vaccine viruses has been criticized, and the plaque assay in cell culture defended, on the grounds that RT-PCR may not detect live infectious viruses but only parts (RNA) of the virus.[2] However, a prolonged presence of the vaccine viruses even as detected by RT-PCR only is an indication of prolonged infection in the intestinal cells and might be associated with clinical consequences, although these are unknown as yet.

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Current Abstract

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Community-Acquired Pneumonia

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Community-acquired pneumonia (CAP) is a syndrome in which acute infection of the lungs develops in persons who have not been hospitalized recently and have not had regular exposure to the health care system. CAP in adults, which remains a major cause of complications and death, is reviewed by the authors.

In the pre-antibiotic era, *Streptococcus pneumoniae* caused 95% of cases of pneumonia. Although pneumococcus remains the most commonly identified cause of CAP, the frequency with which it is implicated has declined, and it is now detected in only about 10–15% of inpatient cases in the United States. In Europe and other parts of the world where pneumococcal vaccines have been used less often and smoking rates remain high, pneumococcus remains responsible for a higher proportion of cases of CAP.

Other bacteria that cause CAP include *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa* and other Gram-negative bacilli. Patients with chronic obstructive pulmonary disease are at increased risk for CAP caused by *H. influenzae*, and *M. catarrhalis*. *P. aeruginosa* and other Gram-negative bacilli also cause CAP in persons who have chronic obstructive pulmonary disease or bronchiectasis, especially in those taking glucocorticoids.

During influenza outbreaks, the circulating influenza virus becomes the principal cause of CAP that is serious enough to require hospitalization, with secondary bacterial infection as a major contributor. Despite the most conscientious efforts to determine the cause, no cause is found in about half the patients who are hospitalized for CAP in the United States, indicating an important area for future investigation.

Scoring systems may predict the severity of disease and help determine whether a patient with CAP requires hospitalization or admission to an intensive care unit. Validated instruments include the Pneumonia Severity Index, the CURB-65 score (a measure of confusion, blood urea nitrogen, respiratory rate, and blood pressure in a patient ≥ 65 years of age) and the guidelines of the Infectious Diseases Society of America and the American Thoracic Society (IDSA/ATS).

Guidelines for empiric antimicrobial therapy for CAP have contributed to a greater uniformity of treatment, and their use in hospitalized patients has been associated with better outcomes. In 2012, the target period from initial contact with the medical care system until antibiotic administration was retired altogether and replaced by the recommendation that treatment be initiated promptly and at the point of care where the diagnosis of pneumonia was first made.

For patients with CAP who require hospitalization and in whom no cause of infection is immediately apparent, IDSA/ATS guidelines recommend empiric therapy with either a beta-lactam plus a macrolide or quinolone alone. These regimens have been studied extensively and generally produce a cure in about 90% of patients with CAP of mild or moderate severity.

A patient whose constellation of findings includes an acute onset of chills and fever, cough with sputum production, pleuritic chest pain, a high or suppressed white count with increased band forms, a dense segmental or lobar consolidation, or a serum procalcitonin level of more than 0.25 μg/L is likely to have typical bacterial pneumonia, such as pneumococcal pneumonia.

Patients with CAP who have none of the factors that favor bacterial infection, and who have known exposure to sick contacts, upper respiratory symptoms at the time of presentation, patchy pulmonary infiltrates, procalcitonin level of 0.1 μg/L or less are unlikely to have bacterial pneumonia.

Comment: Macrolides inhibit important intracellular signaling pathways and suppress production of transcription factors, which decrease the production of inflammatory cytokines and the expression of adhesion molecules. Many, but not all, retrospective studies have shown that the addition of a macrolide to a beta-lactam antibiotic to treat pneumococcal pneumonia or all-cause CAP reduces morbidity and mortality, presumably by inhibiting the inflammatory response.

Statins block the synthesis of 3-hydroxy-methylglutaryl coenzyme A reductase, inhibiting the synthesis of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (which are needed to activate G proteins), thereby dampening inflammatory responses. Observational studies have shown better outcomes in patients who were taking statins at the time of admission for pneumonia. However, no data from randomized trials to examine these effects of macrolides or statins in patients with CAP are available.

The 30-day rate of death in patients who are hospitalized for CAP is approximately 10–12%. After discharge from the hospital, about 18% of patients are readmitted within 30 days. Influenza pneumonia and bacterial pneumonia are each strongly associated with cardiac events.

PUBLICATION II

Shedding of oral pentavalent bovine-human reassortant rotavirus vaccine indicates high uptake rate of vaccine and prominence of G-type G1

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PUBLICATION III

Rotavirus epidemiology 5-6 years after universal rotavirus vaccination: persistent rotavirus activity in older children and elderly

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Rotavirus epidemiology 5–6 years after universal rotavirus vaccination: Persistent rotavirus activity in older children and elderly

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Keywords: Rotavirus, vaccine, RotaTeq

Abstract

***Background*:** Rotavirus (RV) vaccination using RotaTeq® vaccine exclusively was introduced into Finnish National Immunization Program (NIP) in 2009, and soon reached high (≥90 %) coverage. Since mid-2013, all stool samples from laboratory diagnosed cases of RV gastroenteritis in the entire country have been typed.

***Methods*:** 364 RV positive stool samples collected from clinical laboratories over a 2-year period were G- and P-typed using RT-PCR, and the results were confirmed by sequencing. In addition, the genome segment encoding for VP6 was sequenced to distinguish between wild-type and vaccine origin (bovine) RVs.

***Results*:** RV winter epidemic seasons 2013–2014 and 2014–2015 lasted until July each. The age distribution of RV cases showed two unusual clusters: one in children 6–16 years of age, too old to have been vaccinated in NIP, and the other in elderly over 70 years of age. In children diverse genotypes were observed without any obvious predominance. The most common ones were G1P[8] (30.0 %), G2P[4] (22.4 %), G9P[8] (15.8 %), G3P[8] (12.2 %) and G4P[8] (11,2 %). The genotype distribution was not different among vaccinated and unvaccinated children. Most cases in the elderly were associated with G2P[4].

***Conclusions*:** Even at high vaccine coverage and high effectiveness of RV vaccine, RV activity continues to persist, particularly in unvaccinated older children. RV genotypes show greater diversity than before RV vaccinations. We conclude that RV disease can be controlled but not eliminated by vaccinations. Herd-protection in long-term follow-up may be less than at the start of RV vaccinations.

Introduction

Following extensive safety and efficacy trials [1,2], two live oral RV vaccines, Rotarix™ and RotaTeq®, were licensed in 2006. Finland adopted RotaTeq® vaccine exclusively for the National Immunization Programme (NIP) in September 2009, and the coverage of RV vaccination has since been over 90 % (Vaccination register, National Institute for Health and Welfare). The NIP has had high impact on rotavirus gastroenteritis (RVGE), resulting in 90 % reduction of hospitalizations and 90 % of outpatient clinic visits due to RVGE in 2012-2014 compared to pre-licensure years [3]. In May 2013, RV was included in the Finnish Communicable Diseases Act and Decree as part of the microbe strain collection, and all detected RVs in Finland have been referred for typing.

Real-life effectiveness of RV vaccines for RVGE hospitalization in high coverage countries, such as Finland (RotaTeq®) and Belgium (Rotarix™), has been over 90 % [3,4]. In the US, Payne et al. [5] reported 84 % effectiveness of RotaTeq in a 4-year follow-up study with vaccine coverage of 75-80 %. In the US, the peak activity RVGE has become biannual after the introduction of RV vaccines but this has not been observed in Finland or in Austria at higher RV vaccination coverage rates [3,6,7]

While RV vaccinations have shown an indirect impact on RVGE hospitalizations in unvaccinated children and adults in the US and Europe soon after their introduction, in longer follow-up it has been observed that RV activity will persist, and the indirect protection may be less than reported soon after the start of vaccinations[8,9]. We and others have previously reported a shift of RV disease to older unvaccinated children [3,10-12]. In the US, persisting RV disease has also been described in the elderly and in immunocompromised individuals of all ages [13].

RV vaccinations have had some selection pressure on circulating RV strains as new and also unusual genotypes have emerged. In Latin America, particularly in Brazil, G2P[4] became a

predominant genotype for many years after the introduction of Rotarix™ vaccine in NIPs [14,15]. After the introduction of RotaTeq®, a previously unusual genotype G3P[8] and a new genotype G12P[8] have become common in the US and Finland, respectively [5,11]. However, both RV vaccines have remained fully effective against severe RVGE associated with the new genotypes [3,15,16].

Circulating RV strains are constantly monitored and studied, however these studies are usually single- or multi-centered. In this nationwide study we characterize the residual RV activity in Finland, based on routine diagnoses of RVGE in clinical laboratories in years 5 and 6 after the implementation of RV vaccine into NIP.

Materials and methods

In May 2013, 4 years after the introduction of RV vaccination into the Finnish NIP, RV was included in the Finnish Communicable Diseases Act and Decree as part of the microbe strain collection. Since then the surveillance of RV in Finland has included genotyping of all RV positive findings from clinical laboratories around the country. Clinical laboratories may use RV antigen detection with enzyme-linked immunosorbent assay (ELISA) or, more rarely, reverse transcription-polymerase chain reaction (RT-PCR) for primary diagnosis, and positive samples are shipped to National Institute for Health and Welfare (THL). For the period of 2013-2015, these samples were further referred to the Vaccine Research Center, University of Tampere, for genotyping.

Viral RNA was extracted from stool samples using the Qiagen QIAamp Viral RNA Mini Kit (Hilden, Germany) according to the manufacturer's instructions. The RNA extracts were stored in a freezer at -70°C until tested by RT-PCR. RV VP7 and VP4 sequences were detected using a previously described primers and methods [17]. VP6 sequences were detected with a whole-genome PCR method adapted from Matthijnssens et al. [18]. Five µl of RNA was prepared and denatured at 97°C for 2 minutes, otherwise the method was as previously described.

Positive RT-PCR detections were further analyzed by sequencing with the same primers as in RT-PCR. Positive amplicons (VP7, VP4, and VP6) were purified using the Qiagen QIAquick Gel Extraction Kit (Hilden, Germany) and sequenced using the BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM™ 310 Genetic Analyzer. Sequences were analyzed with Sequencher™ 4.10 and compared with published reference strains from GeneBank (http://www.ncbi.nlm.nih.gov/BLAST/, Nucleotide blast).

Statistical analysis was conducted using SPSS 23.0 (IBM SPSS Statistics, IBM Corp.), and p-values between prevalence of genotypes were calculated using Fisher's exact test and Mann-Whitney U test, p-value of < 0.05 was considered as statistically significant.

Because of personal data protection, only limited information from the RV cases was available. This consisted of age with the accuracy of year, gender, sampling date, original result from the clinical laboratory and location of the clinical laboratory which sent the sample to THL. In some cases additional information such as RV vaccination status, travel history, or description of symptoms could be obtained retrospectively.

We arbitrarily divided the cases into four age groups: children aged up to 6 years who were eligible to receive RV vaccine in the NIP, older children aged between 6 and 16 years who were ineligible for the vaccine in NIP, adults aged between 16 and 70 years, and elderly aged 70 years or above.

Results

We received 405 stool samples during the two RV seasons from the beginning of September 2013 to the end of August 2015, covering years 5 and 6 after the start of RV vaccinations in NIP. Of the samples, 172 (42.5 %) were collected during the first RV season (year 5) and 233 (57.5 %) during the second season, 2014-2015 (year 6).

RT-PCR was performed on all 405 samples, and 364 (89.9 %) were found positive for RV VP7. The remaining 41 samples had previously been found to be RV positive by the local clinical laboratories using RV antigen detection. RNA extraction and VP7 RT-PCR were done twice on these apparently false positive samples, but the results remained negative. Also the VP4 and VP6 RT-PCRs of these samples were negative. All 364 VP7 positive samples were positive for RV VP4, and all except for one sample were positive for VP6. Of these 363 samples, human type VP6 was detected in 358 (98.2 %) cases, one had a feline-like origin VP6 (G3P[9]), three had VP6 of bovine origin (G8P[14], G6P[14], and a RotaTeq® vaccine strain), and one sample could not be sequenced for VP6.

Of the 364 RV VP7 positive cases, 303 (83.2 %) were from children to adolescents aged less than 16 years, 44 (12.1 %) from the elderly (aged 70 years or above), and only 17 (4.7 %) cases were from adults aged 16-70 years. Of the 303 children, 160 (52.8 %) were aged between 0 to 6 years of age and were considered as eligible to have received RV vaccination in the NIP. The remaining 143 (47.2 %) children were from 6 to 16 years of age, and therefore too old to have received the vaccine in the NIP.

Of all 364 RV positive samples, 160 (44.0 %) were collected during the first RV season and 204 (56.0 %) during the second RV season. The monthly distribution of RV cases during the two seasons is shown in Figure 1. In both years, RV activity continued late until July. The seasonality in adults and elderly was not different from that in children.

Genotypes

In total, G2P[4] was the most common genotype as it was detected in 112 (30.8 %) of the 364 VP7 positive cases. The second most common genotype was G1P[8] with 98 (26.9 %) detections. Other common genotypes were G9P[8] with 48 (13.2 %) and G3P[8] with 41 (11.3 %) cases. G12P[8] was detected in 19 (5.2 %) samples. Other genotypes were less frequent and are presented in Table 1. [Table 1 near here]

In children (n=303), in both 0-6 years and 6-16 years, G1P[8] was the most predominant genotype with 91 (30.0 %) detections during both seasons combined. However, in the 0-6 year olds G9P[8] was more frequently detected than G1P[8] during the second season 2014-2015. In adults (n=17) and the elderly (n=44), G2P[4] was detected most frequently with 11 (64.7 %) and 33 (75.0 %) cases, respectively. There were only minor changes in the genotypes between these two seasons. The proportion of G9P[8] increased from 8.3 % to 22.7 % in the 0-6 year olds and from 10.5 % to 18.6 % in 6-16 year olds from the first to the second season. During the same period G4P[8] decreased from 23.6 % to 6.8 % in the 0-6 year olds. (Table 1)

Genotypes in vaccinated and unvaccinated children

Of the 303 children, 29 (9.6 %) were known to have received at least one dose of rotavirus vaccine, 80 (26.4 %) had not received vaccine, and the vaccination status of the rest, 194 (64.0 %) children, was unknown. We estimated that from those 80 unvaccinated children, 31 were eligible to have received the vaccine according to their age, and therefore 49 children were unvaccinated and ineligible to have received the vaccine. G1P[8], G9P[8] and G12P[8] were seen more often in vaccinated children (24.1 %, 20.7 % and 13.8 % in comparison to 16.1 %, 12.9 % and 6.5 %, respectively, in unvaccinated children) whereas G2P[4], G3P[8] and G4P[8] were more frequently detected in unvaccinated children (10.3 %, 3.4 % and 20.7 % in comparison to 19.4 %, 12.9 % and 29.0 %, respectively). However, the differences in

genotypes between unvaccinated and vaccinated children were not statistically significant (p=0.425, Mann-Whitney U test). G4P[8] was the only genotype more frequently detected in the unvaccinated eligible than in unvaccinated ineligible older children (29.0 % vs. 10.4 %, respectively); the difference was statistically significant, p=0.039 (Fisher's exact test). (Figure 2)

Discussion

We conducted a nationwide study on RV detections in patients of all age during two RV seasons, 2013-2014 and 2014-2015, in years 5 and 6 after the introduction of RotaTeq® vaccine into the NIP in Finland.

This study showed that RV continues to circulate and cause clinical disease despite high vaccination coverage (90-95 %) and high effectiveness (94.4 %) against RV hospitalizations in Finland [19]. It may be speculated that the RV activity is maintained from an unvaccinated children population not eligible for RV vaccination in NIP started in 2009. We noted a cluster of RV activity in children aged 6 to 16 years, too old to have received RV vaccine in NIP. Also in Belgium, a country with long-term use of Rotarix™ at high coverage, RV activity has remained at the same level from years 4 to 6 after implementation of RV vaccine, and has been hypothesized to be maintained from a source outside of the vaccinated population [12].

We also noted a second age cluster of RVGE in people 70-90 of age. RVs in adults might form a reservoir which contributes to continuing RV activity in children. This speculation is also supported by a study of Prelog et al. [7] from Austria who noted that as the RV activity in newborns has not decreased even after several years of universal mass vaccination, there must be an unknown reservoir of RV which has frequent contacts with the newborns. RV activity in the elderly is of interest since it may follow its own epidemiology as reported from Japan where the RV activity in adults was observed all-year-round [20]. RV disease in adults as a separate entity is also supported by the finding that the RV vaccines have no herd-effect on the disease in the adults outside epidemic season [21]. Clearly, continued surveillance of RV disease in adults is required to obtain a full view of the remaining RV activity in the population. RV genotype distribution in adults has previously been found to be broader than in children with G2P[4] usually the most predominant genotype [8,22]. Our results were similar: G2P[4] was the most common genotype in both seasons.

Various zoonotic strains were detected in adults and children. The G8P[14] and G9P[6] genotypes were detected for the first time in Finland; G8P[14] has been previously detected also in the US [23] and Mali [24], and G9P[6] in the Netherlands [25] and India [26]. Also genotype G8P[8], which is globally more common, was detected for the first time in Finland.

In Finland before RV vaccine was added to the NIP, G1P[8] was the predominant genotype causing more than 60 % of the RV cases [17]. In the US G1P[8] accounted for 78.5 % of all RV infections between the years 1996 and 2005 [27]. After introduction of RV vaccination G2P[4] and G3P[8] have become predominant RV genotypes in Rotarix™ (G2P[4]) and RotaTeq® (G3P[8]) using countries such as Belgium and some territories of Australia, respectively [28,29]. Interestingly, in Finland the effect of RotaTeq® vaccine on circulating RV strains was not so clear as G1P[8] continued to predominate in the first years of NIP[3,11,17], while also G3P[8], G4P[8], and G12P[8] emerged [30]. The present findings in years 5 and 6 after NIP confirm the continued presence of G3P[8], G4P[8], and G12P[8], and G9P[8] becoming common in year 6. Altogether, the genotype diversity we observed in this study has not been reported before to such an extent in a country with high vaccine coverage and vaccine effectiveness of RV vaccine.

Interestingly, there was no major difference in RV genotype distribution of vaccinated and unvaccinated children. This suggests that the genotype specificity of RotaTeq® vaccine is not critical for protection against severe RV disease, and breakthrough cases may be caused by a multitude of RV genotypes. This is consistent with the hypothesis that most of the protection against severe RVGE is mediated by non-neutralizing antibodies against RV VP6 [31,32].

We have recently shown that shedding of RotaTeq® vaccine viruses into stools is common [33]. In this study, only one child in the susceptible age group (under one year of age) was detected with RotaTeq® vaccine-derived double-reassortant G1P[8] (vdG1P[8]) RV in stool specimen. VdG1P[8] is linked with more severe symptoms which may lead to hospitalization[30] whereas the single reassortant vaccine strains do not have similar

virulence and are usually shed without symptoms [30,33]. The low detection rate of vaccine strains in our study may be because most specimens from children with RVGE were mostly from hospitalized or otherwise severe cases, and cases with mild gastrointestinal symptoms would not be referred to laboratory studies. Severity of the cases is discussed below.The high number of false-positive clinical laboratory RV findings is remarkable. However, as most of the clinical laboratories in Finland use commercial ELISA RV antibody detection kits, which have the specificity of 80-90 %, our detection rate of false-positive findings (around 10 %) is not out of line [34]. Bowen et al. [6] described a similar proportion (around 15 %) of false-positive RV samples while analyzing ELISA positive samples with RT-PCR and qRT-PCR.

The true number of RV cases in children is probably higher as clinically milder cases are usually taken care without laboratory diagnostics. In the adults and elderly RV is rare and is not tested routinely. Therefore, it can be speculated that most of the cases have had severe RVGE which has led to healthcare contact and diagnostic testing. Our study was nationwide and took advantage of the recent legislation that every clinical laboratory (public or private) is required to send all positive RV samples for further typing. The major limitation of our study was the limited clinical information. We did not receive medical history, vaccine status, clinical picture, or travel history in most cases. This was due to two reasons. Firstly, the referral form by the clinicians was free narrative and therefore was often left unfilled. Secondly, the privacy of health information is strictly defined in the Finnish legislation and due to the epidemiological nature of our study our access to medical histories was limited. Despite the limitation of incomplete clinical information available to us, a strength of the study is that we likely collected all RV cases which were diagnosed in Finland during these two RV seasons, including cases in adults and the elderly, and therefore the material is exceptionally comprehensive. In 2014 Lawrence et al. [35] reported on the safety and immunogenicity of RotaTeq® vaccine in the elderly with promising results. The vaccine increased RV IgA levels even after one dose, and was well tolerated. In the light of our study,

the burden of disease caused by RV in the elderly is small and perhaps not sufficient to warrant a standalone RV vaccination. However, as part of combined norovirus-RV vaccine currently under development also the RV component might be of significance and add the value of such a vaccine in the elderly [36].

In conclusion, residual RV activity is persisting 6 years after RV vaccine in NIP despite the high vaccine coverage and effectiveness. Our results show that the RV activity is shifting towards older unvaccinated children, while also being maintained in the elderly population. Furthermore, probably under vaccine-induced immune pressure, the diversity of RV genotypes in the remaining RV cases has increased. We conclude that RV disease can be controlled but not eliminated by vaccination with live oral RV vaccines.

Disclosure of interest

Timo Vesikari has been PI of clinical trials of rotavirus vaccines produced by Merck and GlaxoSmithKline, and has also been a member of an advisory board of Sanofi Pasteur-MSD. Other authors report no conflicts of interest.

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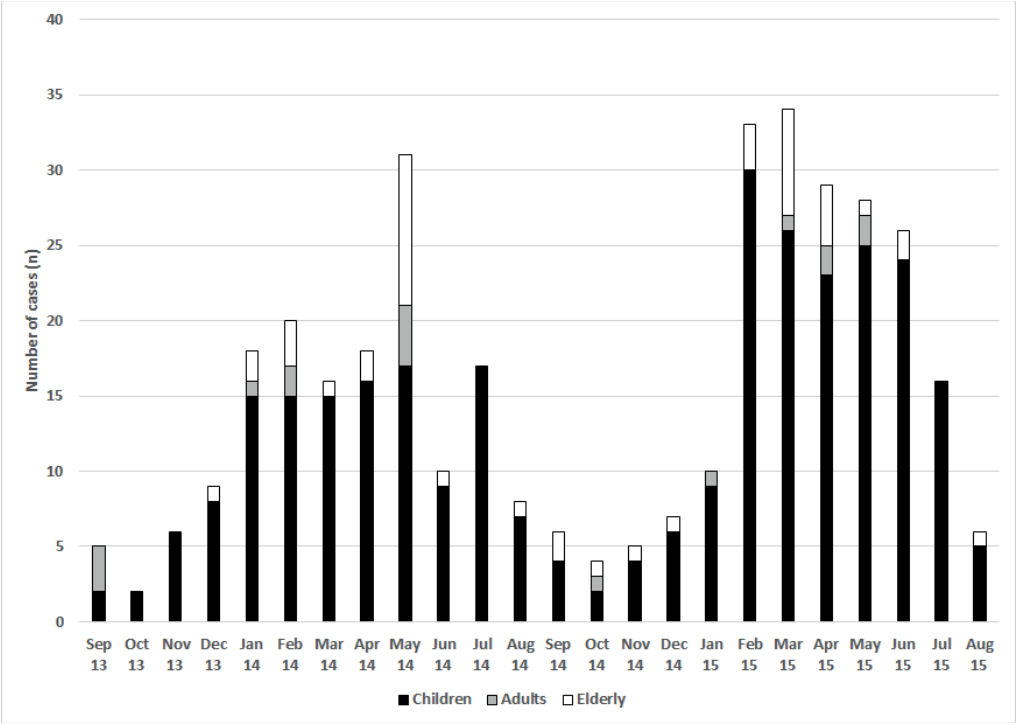

Figure 1. Seasonality of RV cases (n=364) in children, adults and elderly from September 2013 to August 2015.

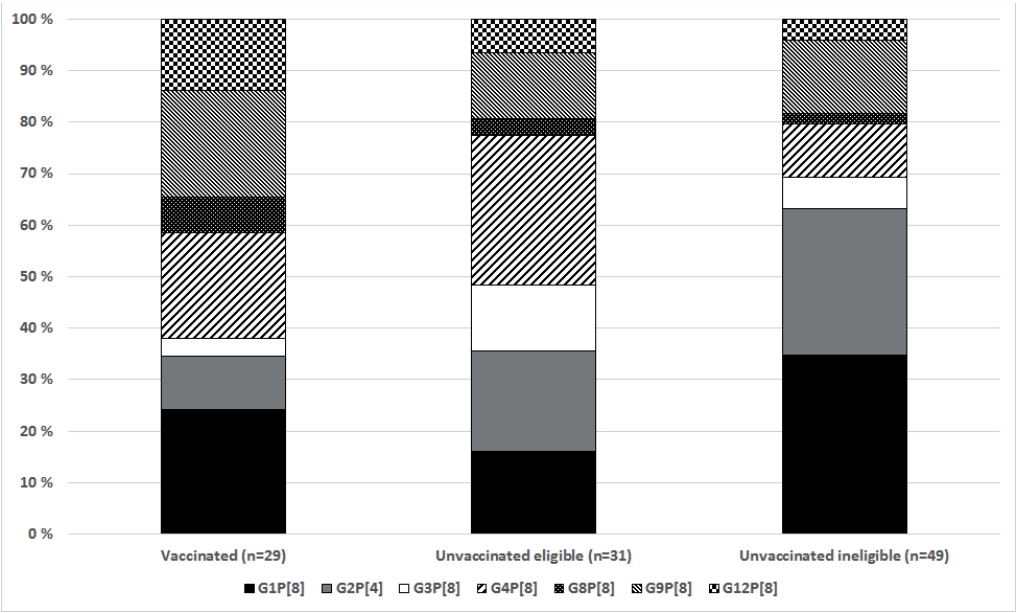

Figure 2. Proportion of RV genotypes detected in vaccinated, unvaccinated RV vaccine eligible and unvaccinated RV vaccine ineligible children in years 5 and 6 after implementation of RV vaccination with RotaTeq® vaccine.

Table 1. Detection rates of rotavirus genotypes in different age groups during two RV seasons, 2013-2014 and 2014-2015, or years 5 and 6 after RV vaccine (RotaTeq®) in NIP. Predominant genotypes in each season are bolded.

	No of detections, n (%)								
Age group	0-6 y		6-16 y		16-70 y		70- y		
Season	2013-2014	2014-2015	2013-2014	2014-2015	2013-2014	2014-2015	2013-2014	2014-2015	*Total, n (%)*
Genotype									
G1P[8]	**21 (29.2)**	18 (20.5)	**23 (40.4)**	**29 (33.7)**	1 (12.5)	2 (22.2)	1 (4.8)	3 (13.0)	98 (26.9)
G2P[4]	14 (19.4)	19 (21.6)	12 (21.1)	23 (26.7)	**6 (75.0)**	**5 (55.6)**	**18 (85.7)**	**15 (65.2)**	**112 (30.8)**
G3P[8]	9 (12.5)	15 (17.0)	6 (10.5)	7 (8.1)	0	0	1 (4.8)	3 (13.0)	41 (11.3)
G3P[9]	0	0	0	0	1 (12.5)	0	0	0	1 (0.3)
G4P[8]	17 (23.6)	6 (6.8)	7 (12.3)	4 (4.7)	0	0	1 (4.8)	1 (4.3)	36 (9.9)
G6P[14]	0	0	0	0	0	1 (11.1)	0	0	1 (0.3)
G8P[8]	2 (2.8)	0	0	2 (2.3)	0	0	0	0	4 (1.1)
G8P[14]	0	0	1 (1.8)	0	0	0	0	0	1 (0.3)
G9P[6]	0	0	0	1 (1.2)	0	1 (11.1)	0	0	2 (0.5)
G9P[8]	6 (8.3)	**20 (22.7)**	6 (10.5)	16 (18.6)	0	0	0	0	48 (13.2)
G12P[8]	2 (2.8)	10 (11.4)	2 (3.5)	4 (4.7)	0	0	0	1 (4.3)	19 (5.2)
RotaTeq®	1 (1.4)	0	0	0	0	0	0	0	1 (0.3)
Total, n	72	88	57	86	8	9	21	23	364

PUBLICATION IV

Continuing rotavirus circulation in children and adults despite high coverage rotavirus vaccination in Finland

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